A Role for the Ubiquitin Domain Protein HERP in ER-associated Protein Degradation

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I

der Humboldt-Universität zu Berlin

von

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geboren am 10.03.1976 in Brilon

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Tag der mündlichen Prüfung: 27. September 2006

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.

Thomas Henry Huxley

ABSTRACT

ER-associated protein degradation (ERAD) is part of the ER quality control system dealing with the accumulation of misfolded proteins in the ER. This process requires polyubiquitylation of ERAD substrates involving E3 ligases, such as HRD1, and their subsequent extraction from the ER membrane by the p97-Ufd1-Npl4 complex. Retrotranslocation of substrates into the cytosol for degradation by the 26S proteasome also involves the membrane proteins Derlin-1 and VIMP, which are associated with p97 to form a protein complex.

The ER-resident protein HERP was shown to be upregulated by the unfolded protein response pathway (UPR) upon the accumulation of misfolded proteins in the ER. It was therefore considered to function in ERAD. Interestingly, HERP contains a UBL domain. In other proteins this domain facilitates an interaction with the proteasome, suggesting that HERP might recruit the proteasome to the ER membrane for efficient ERAD. The aim of this study was to investigate the function of HERP within the UPR.

The findings presented here demonstrate that HERP is essential for the degradation of a model ERAD substrate. Thus, HERP indeed has a role in ERAD. Moreover, the data show that HERP directly interacts with the E3 ligase HRD1 and the two proteins form a common protein complex with p97, Derlin-1 and possibly also with VIMP. This suggests that both ubiquitylation and retrotranslocation of ER proteins are performed by one protein complex, enabling an efficient processing of ERAD substrates.

This study also demonstrates that the UBL domain of HERP does not share the proteasome binding property of other UBL domains, suggesting that proteasome binding cannot be considered a general feature of all UBL domains. Instead, the HERP UBL domain is able to interact with the deubiquitylating enzyme USP7. Therefore, deubiquitylation might also be an important aspect in the proteasome-dependent degradation of misfolded ER proteins.

ZUSAMMENFASSUNG

Die ER-assoziierte Proteindegradation (ERAD) ist Teil des Qualitätskontrollsystems am ER, um der Akkumulation von fehlgefalteten Proteinen im ER entgegenzuwirken. Hierbei werden ERAD-Substrate mit Hilfe von E3-Ligasen wie z.B. HRD1 ubiquityliert und anschließend durch den p97-Ufd1-Npl4 Komplex aus der ER-Membran extrahiert. Im Zytosol werden diese extrahierten Proteine vom 26S Proteasom abgebaut. Für die Retrotranslokation von ERAD-Substraten werden zudem die Membranproteine Derlin-1 und VIMP benötigt, welche mit p97 assoziieren und einen Proteinkomplex bilden.

HERP ist ein ER-lokalisiertes Protein, dessen Synthese durch den UPR (unfolded protein response) als Antwort auf die Akkumulation von fehlgefalteten Proteinen im ER induziert wird. Dies deutet auf eine Rolle von HERP im ERAD hin. Interessanterweise besitzt HERP eine sogenannte UBL-Domäne. Für andere Proteine mit UBL-Domäne konnte eine Interaktion dieser Domäne mit dem Proteasom nachgewiesen werden. Daher kann angenommen werden, dass HERP ebenfalls mit dem Proteasom interagiert und dies zur ER-Membran rekrutiert, wo es für den Abbau von ERAD-Substraten benötigt wird. Das Ziel der vorliegenden Arbeit war es, die Rolle von HERP innerhalb des UPR zu ermitteln.

Die hier präsentierten Daten zeigen, dass HERP essentiell für den Abbau des ERAD-Modell-Substrates CD3-delta ist. Somit hat HERP tatsächlich eine Rolle im ERAD. Außerdem wird eine direkte Interaktion von HERP mit der E3-Ligase HRD1 nachgewiesen. Es wird zudem gezeigt, dass HERP und HRD1 einen Proteinkomplex mit p97, Derlin-1 und eventuell auch mit VIMP bilden. Dieser ERAD Komplex ist folglich sowohl für die Ubiquitylierung als auch die Retrotranslokation von ERAD-Substraten verantwortlich und garantiert somit die effiziente Prozessierung von Proteinen aus dem ER.

Zudem wird gezeigt, dass die UBL-Domäne von HERP im Gegensatz zu anderen UBL-Domänen nicht mit dem Proteasom interagiert. Somit kann nicht mehr davon ausgegangen werden, dass Proteasombindung eine Gemeinsamkeit aller Proteine mit UBL-Domäne ist. Dagegen wird eine Interaktion der UBL-Domäne von HERP mit dem deubiquitylierenden Enzym USP7 nachgewiesen. Dies deutet darauf hin, dass auch Deubiquitylierung eine wichtige Rolle im ERAD-Prozess spielt.

Keywords

HERP, HERPUD1, UBL domain, ubiquitin-like domain, ubiquitin domain protein, UPR, endoplasmic reticulum, ERAD, ubiquitin, ubiquitylation/ubiquitination, retrotranslocation, protein degradation, proteasome, HRD1, p97/VCP/Cdc48, Derlin-1, VIMP, USP7, protein complex.

Schlagworte

HERP, HERUD1, UBL-Domäne, Ubiquitin-ähnliche Domäne, Ubiquitin Domänen Protein, UPR, Endoplasmatisches Retikulum, ERAD, Ubiquitin, Ubiquitylierung/Ubiquitinierung, Retrotranslokation, Proteindegradation, Proteasom, HRD1, p97/VCP/Cdc48, Derlin-1, VIMP, USP7, Proteinkomplex

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1 INTRODUCTION

1.1 The ubiquitin-proteasome system

Cellular protein levels are determined not only by the rates of synthesis but also by the rates of degradation. The life span of intracellular proteins varies widely, from minutes to days, and differential rates of protein degradation are an important aspect of cell regulation. The major pathway for selective protein degradation is the ubiquitin-proteasome system. It mediates the proteolysis of many regulatory proteins making it central in the control of numerous processes including cell-cycle progression, signal-transduction, transcriptional regulation, development, apoptosis and antigen processing. It is also essential for the breakdown of damaged and misfolded proteins to prevent their accumulation in the cell (Hershko and Ciechanover, 1998).

The discovery of the ubiquitin-proteasome system began in 1978 when a heat-stable polypeptide was found to be required for the activity of an ATP-dependent proteolytic system in reticulocytes (Hershko et al., 1979). This polypeptide was identified as ubiquitin, a highly conserved protein present in all eukaryotes (Wilkinson et al., 1980). Subsequently, Hershko and Ciechanover discovered that ubiquitin is covalently linked to protein substrates in an ATP-dependent manner (Ciechanover et al., 1980). This process was termed ubiquitylation. The presence of a chain of substrate linked ubiquitins recruits the 26S proteasome for degradation. The 26S proteasome is a 2.5 MDa complex that uses the energy derived from ATP to unfold the protein substrate and translocate it into its interior chamber where it is degraded (Baumeister et al., 1998). Thus, ubiquitin-proteasome mediated breakdown of proteins occurs in two distinct steps. First, polyubiquitin chains are covalently attached to the targeted protein and second, the polyubiquitylated protein is degraded by the 26S proteasome (Fig. 1A) (Pickart, 2004).

1.1.1 The ubiquitin conjugation cascade

The attachment of ubiquitin to an acceptor protein requires the activity of three classes of enzymes called E1, E2 and E3 (Fig. 1B). These enzymes react sequentially with ubiquitin in a conjugation cascade, finally resulting in the transfer of ubiquitin onto the acceptor protein. First, ubiquitin is activated by a ubiquitin activating enzyme (E1) in an ATP-consuming process, in which a thiol ester between the C-terminus of ubiquitin and the E1 is formed. Second, the activated ubiquitin molecule is transferred to a cysteine side chain of a conjugating enzyme (E2). In a final step, a ubiquitin ligase (E3) associates with the E2 and transfers the activated ubiquitin

molecule from the E2 to the substrate protein, mostly to the ε-amino-group of a lysine side chain (Hershko et al., 1983). This conjugation cascade can act repeatedly on a single acceptor protein, resulting in the formation of ubiquitin chains. Such chains are formed by isopeptide linkages between a lysine side chain of the proximal ubiquitin with the glycine residue at the carboxyl terminus (G76) of the distal ubiquitin moiety. Occasionally polyubiquitylation of substrates requires an additional enzyme for the elongation of ubiquitin chains. These so-called E4 enzymes recognise oligoubiquitylated substrates and perform polyubiquitylation in cooperation with E1, E2 and E3 (Hoppe, 2005). The best characterised E4 enzymes, Ufd2 and CHIP, contain a U-box, which is involved in the elongation of oligomeric ubiquitin chains (Koegl et al., 1999). Interestingly, E4s can also have E3 activity as will be discussed later.

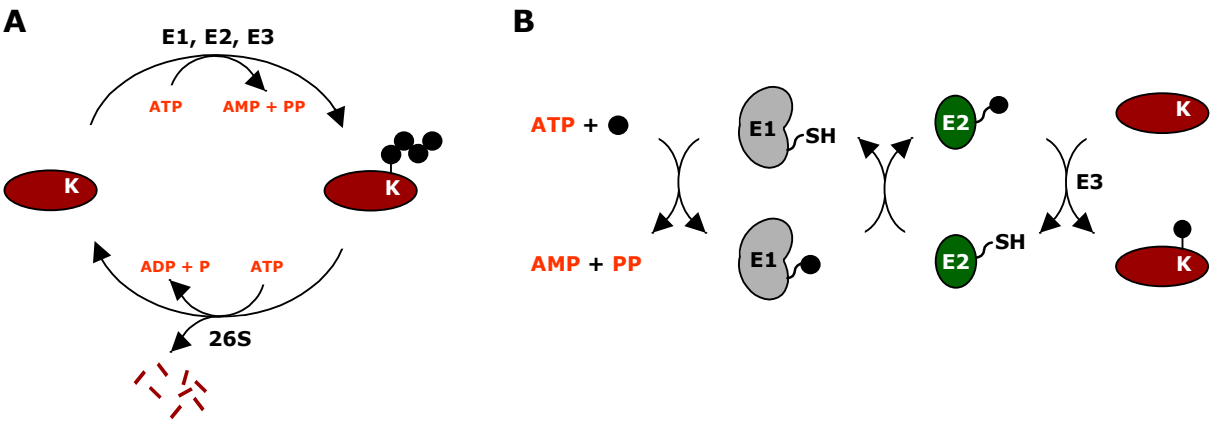

Figure 1. Mechanism of the ubiquitin-proteasome pathway. (A) Overview of the pathway with its conjugative (top) and degradative (bottom) phases. **(B)** Enzymatic pathway of ubiquitin conjugation. E1, E2 and E3 are ubiquitin activating enzyme, ubiquitin conjugating enzyme and ubiquitin ligase, respectively. Black circles indicate ubiquitin and K the lysine residue of the substrate (dark red). ATP is used for ubiquitylation and degradation of the substrate (adapted from Pickart, 2004).

The organisation of the ubiquitin conjugation cascade is hierarchical. In humans there is only one single and essential E1 enzyme known so far, which activates all ubiquitin molecules. The activated ubiquitins are then transferred to one of about 30 different E2 enzymes. These E2s then serve a much larger number of E3s. Each E3 enzyme cooperates with only one or a few E2s and simultanously specifically interacts with the protein substrate. Thus, substrate specific E3 ligases provide an element of regulation to the ubiquitylation process (Pickart, 2001).

1.1.2 E3 ligases

The human genome contains hundreds of genes encoding E3 ubiquitin ligases. These can be divided into RING E3s (Really Interestig New Gene), U-box E3s (Ufd2-homology) and HECT E3s (Homologous to E6AP Carboxy Terminus). The RING E3s are by far the largest family of E3 ligases. These enzymes contain a conserved motif rich in cysteine and histidine residues, which coordinate two zinc ions. This motif called RING domain is responsible for the enzymatic activity. Many RING domains have been shown to interact directly with E2 enzymes (Fig. 2) (Brzovic et al., 2003; Lorick et al., 1999) and there is evidence that this interaction is important for the biological function of the E3 RING domain (Wu et al., 2003).

RING E3s exist either as single proteins or as complexes with multiple subunits (Fig. 2A, B). Single subunit E3s such as HRD1 or parkin contain a substrate recognition element and a RING finger domain on the same polypeptide chain (Bays et al., 2001; Imai et al., 2001; Imai et al., 2000). Multisubunit E3 ligases, on the other hand, consist of a protein of the cullin family serving as backbone for the complex, a RING finger protein for ligase activity and other proteins, some of which are involved in substrate recognition. An SCF complex (SKP1-Cullin-F-box-protein), for instance, contains the proteins SKP1, Cullin-1, RBX1 and an F-box protein. Cullin-1 functions as a modulator scaffold that simultaneously interacts with the RING finger protein RBX1 and the adaptor protein SKP1. RBX1 harbours the catalytic activity for the transfer of ubiquitin from the E2 to the substrate protein, whereas the adaptor protein SKP1 recruits an F-box protein to the complex (Fig. 2B). F-box proteins are responsible for substrate recognition. They target phosphorylated substrate proteins, e.g. G1 cyclins, β-catenin or Iκ-Bα, and deliver them to the SCF-complex for their ubiquitylation. Eukaryotic cells contain many different F-box proteins, all of which are responsible for the specific interaction of the SCF complex with a substrate protein. Through the exchange of F-box proteins different substrates can be recruited to the complex for ubiquitylation. Hence, multisubunit E3s give an example of how substrate specificity in ubiquitin conjugation is achieved through combinatorial mechanisms (Pickart, 2001; Weissman, 2001).

The U-box defines a rather small number of E3 ligases. Like the RING domain, the U-box is involved in E2 binding and catalysis. It was initially identified in Ufd2, a protein that was described as an E4 enzyme (Koegl et al., 1999). First evidence that this domain can also act as an E3 enzyme came from biocomputational analyses revealing that the structures of the U-box and the RING domain are similar (Aravind and Koonin, 2000). Experimental data showing that the U-box directly interacts with E2s and that U-box proteins can ubiquitylate themselves later

confirmed this finding (Hatakeyama et al., 2001). CHIP is another example for a well described U-box protein with E3 ligase activity. It functions in cooperation with the chaperones Hsp70 and Hsp90 and has been described to be responsible for the ubiquitylation and thereby degradation of important disease-related proteins such as the cystic fibrosis transmembrane receptor (CFTR) (Meacham et al., 2001), the glucocorticoid receptor (Connell et al., 2001) and the tau protein (Petrucelli et al., 2004; Shimura et al., 2004).

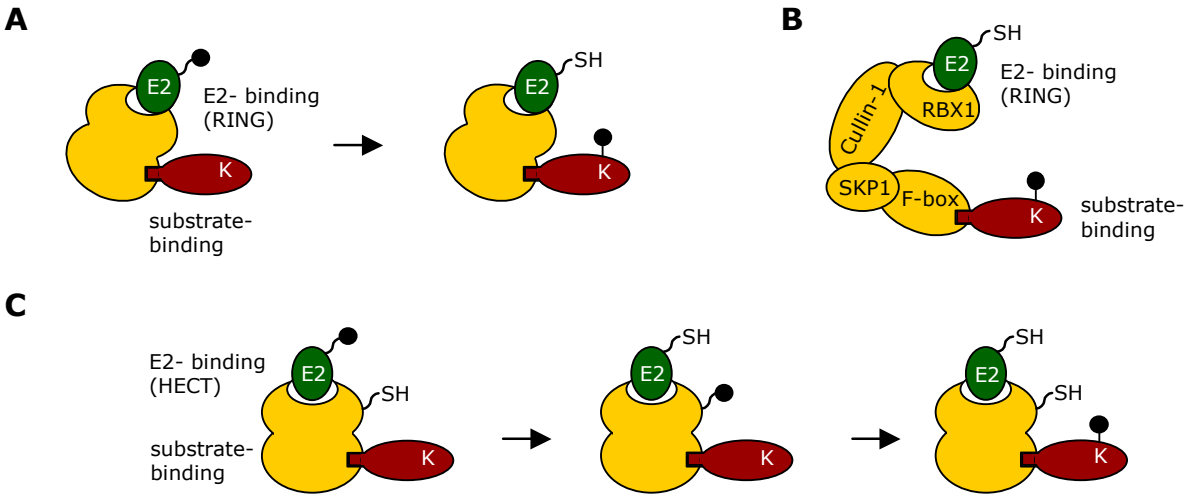

Figure 2. Major E3 classes. RING domain E3s (yellow) appear as either **(A)** single subunit E3s or as **(B)** multisubunit E3s. They use the RING domain to bind the E2 and another domain or in the case of multisubunit E3s another protein such as an F-box protein to bind the substrate (dark red). **(C)** HECT-domain E3s (yellow) bind E2s via the HECT domain. The ubiquitin is transiently bound to the E3 before it is transferred onto the substrate. Black filled circles indicate ubiquitin and K the lysine residue of the substrate. (adapted from (Pickart, 2004).

RING E3s and U-box E3s act as bridging factors, directly transferring ubiquitin from the E2 enzyme onto the substrate. The third class of E3 ligases are the HECT E3s (Homologous to E6AP Carboxy Terminus). They employ a mechanism unique among the ubiquitin ligases by forming a thiol ester intermediate with ubiquitin (Fig. 2C). The first and eponymous member of this family is E6AP (Scheffner et al., 1993). Upon binding to the human papilloma virus protein E6 it ubiquitylates p53 and therefore targets it for degradation. The N-terminus of E6AP mediates substrate binding, whereas the HECT domain binds the E2. Ubiquitin is then transferred to a conserved cysteine residue within the HECT domain of E6AP before it is passed on to the substrate (Huibregtse et al., 1995).

1.1.3 Diverse functions of ubiquitylation

Ubiquitylation serves different functions in the cell. Apart from its role in protein degradation by the 26S proteasome, it also targets proteins to lysosomes via endocytosis. Additionally, ubiquitylation is involved in the regulation of nonproteolytic cellular processes such as ribosomal function, postreplicational DNA repair and transcription (Pickart, 2001).

The way in which ubiquitin is linked to proteins is crucial to the fate of a substrate protein. A substrate protein can be modified with one or more single ubiquitin molecules or with one or more polyubiquitin chains, resulting in mono- or polyubiquitylation, respectively. Only polyubiquitin chains target proteins for proteasomal degradation. However, different lysine residues within ubiquitin can be used for chain formation. Ubiquitin has seven lysine residues of which K11, K29, K48 and K63 can form ubiquitin-ubiquitin linkages *in vivo* (Dubiel and Gordon, 1999). K48-G76 linked chains of four or more ubiquitins are potent targeting signals that lead to recognition and degradation of substrate proteins by the 26S proteasome (Chau et al., 1989; Thrower et al., 2000). In contrast, polyubiquitin chains with K63-G76 isopeptide bonds are not proteasome targeting signals. Instead, they have been implicated in DNA-repair, the activation of IκB kinase and other cellular processes (Spence et al., 1995; Deng et al., 2000). Monoubiquitylation on the other hand has multiple functions other than protein degradation, e.g. receptor internalisation or regulation of transcription (Hicke, 2001).

1.1.4 The 26S proteasome

Once a substrate protein has been modified with a K48-G76 linked chain with at least four ubiquitin moieties, it can be degraded by the 26S proteasome (Thrower et al., 2000). This multisubunit protease consists of the catalytically active 20S core particle and one or two 19S regulatory particles (Fig. 3). The 20S core forms a cylindrical structure composed of four stacked rings, each of them containing seven evolutionary related proteins (Groll et al., 1997). These proteins can be subdivided into two groups based on their relative sequence similarities, the α- and β-subunits. The two inner rings are composed of the β-subunits β1 to β7. They enclose a central chamber harbouring the active sites. Access to this central chamber is provided by the outer α-rings, composed of α1 to α7 (Groll et al., 2000). A 19S complex is associated with either one or both ends of the 20S core and controls the entry of substrate proteins. This 19S regulatory complex can be dissociated into two subcomplexes called base and lid. The base is formed by a ring of six ATPase subunits, which contact the 20S core, as well as S1/Rpn2 and S2/Rpn1, the

two largest subunits of the proteasome. It is responsible for binding and unfolding of substrate proteins as well as their translocation into the 20S core (Braun et al., 1999; Lam et al., 2002). The lid is involved in deubiquitylation of substrate proteins prior to their degradation (Verma et al., 2002). It consists of eight subunits. The S5a/Rpn10p subunit is located in the hinge area between base and lid and contributes to their stable association (Glickman et al., 1998). S5a/Rpn10p has also been found as a free form that binds ubiquitin conjugates (Deveraux et al., 1994).

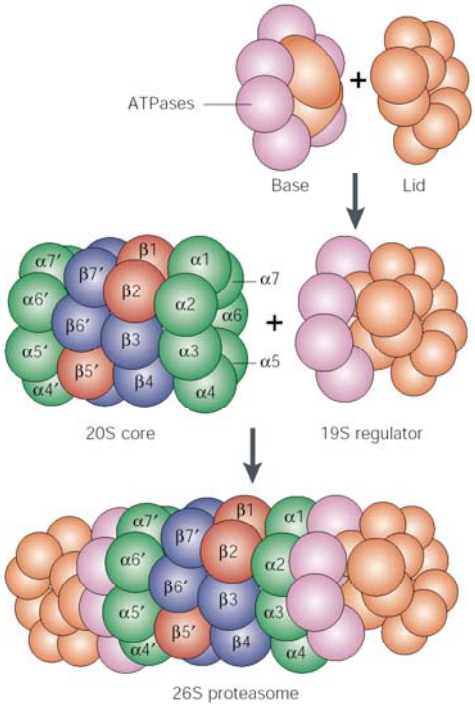

Figure 3. The 26S proteasome. The 26S proteasome consists of the 20S core particle and the 19S regulatory particles. The 20S core particle forms a cylindrical structure of four heptameric rings. The two inner rings consist of the seven β-subunits, of which β1, β2 and β5 harbour the active sites. The two outer rings consist of α subunits. The 19S regulator can be dissociated into two sub-complexes, the base, which is involved in substrate recognition, and the lid, which was found to serve as a receptor for ubiquitin conjugates. S5a/Rpn10p binds to both subcomplexes and promotes their stable association. Additionally, it serves as a soluble receptor for ubiquitin conjugates (from Kloetzel, 2001).

1.1.5 Deubiquitylating enzymes

Ubiquitin-protein conjugates are highly dynamic structures. While an array of enzymes directs the conjugation of ubiquitin to substrates, there are also dozens of deubiquitylating enzymes (DUBs) that can reverse this process. These enzymes remove ubiquitin from substrate proteins by cleaving ubiquitin-linked molecules after the terminal carbonyl of the last residue of ubiquitin (G76). DUBs are important regulators of the ubiquitin-proteasome system. One function of DUBs is to release ubiquitin chains from ubiquitin-protein conjugates once they have been targeted to the proteasome and committed to degradation. These DUBs are either proteasome subunits themselves, like Uch37 and Rpn11, or associate with the 26S proteasome to mediate

substrate deubiquitylation (Borodovsky et al., 2001; Lam et al., 1997; Leggett et al., 2002; Papa et al., 1999; Verma et al., 2002). By removing ubiquitin from proteasomal substrates, these DUBs are also thought to help maintaining the level of free ubiquitin, which can thereby be recycled in a new ubiquitylation cascade (Amerik and Hochstrasser, 2004).

Apart from their function in ubiquitin recycling at the proteasome DUBs are also essential for the activation of ubiquitin precursors. Ubiquitin is synthesised as an inactive precursor with a C-terminal extension to prevent premature conjugation. These extensions are removed by DUBs to activate ubiquitin (Amerik and Hochstrasser, 2004). Moreover, DUBs can regulate protein degradation negatively by removing or trimming polyubiquitin chains from substrate proteins prior to their delivery to the proteasome. The DUB USP7, for instance, has been implicated in the deubiquitylation of p53, a tumor suppressor protein that is mutated in many cancers (Levine, 1997). Normally, p53 is a short lived protein, which is rapidly degraded by the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). The DUB USP7 specifically interacts with p53 and deubiquitiylates the protein. This results in the stabilisation of p53 leading to an inhibition of cell proliferation (Li et al., 2002). Thus, USP7 can specifically regulate p53 levels by its deubiquitylation and might therefore act as a tumor suppressor protein.

1.2 Ubiquitin domain proteins (UDPs)

1.2.1 Ubiquitin and ubiquitin-like proteins

Ubiquitin is a phylogenetically highly conserved protein, as only three out of its 76 amino acid residues differ between mammals, yeast and plants. It has a highly stable and compact structure consisting of two α-helices and five β-sheets. The helical and β-sheet structures arrange in the order ββαββαβ, forming the ubiquitin superfold (Vijay-Kumar et al., 1987). During the last decade a number of proteins related to ubiquitin have been identified. All of these contain a ubiquitin superfold and most of them also resemble ubiquitin in terms of their primary sequence and three-dimensional structure. Ubiquitin-like proteins can be divided into two groups according to their function: ubiquitin-like modifiers and ubiquitin domain proteins (Jentsch and Pyrowolakis, 2000).

Ubiquitin-like modifiers comprise a group of small proteins including ubiquitin itself, NEDD8, SUMO, FAT10 and others that act as post-translational modifiers analogous to

ubiquitin. Their covalent attachment to target proteins requires enzymes closely related to the E1, E2 and E3 enzymes found in the ubiquitin pathway. Most ubiquitin-like modifiers are synthesised as inactive precursors with C-terminal extensions to prevent unwanted conjugation. These extensions are endoproteolytically clipped off leading to conjugation-competent ubiquitin-like modifiers terminating in a glycine or diglycine (Jentsch and Pyrowolakis, 2000). Despite their structural similarity, different ubiquitin-like modifiers appear to be involved in diverse cellular functions. The SCF complex, for instance, the multisubunit E3 ligase mentioned earlier, is activated by covalent attachment with the ubiquitin-like modifier NEDD8, which participates in cell cycle control, signalling and embryogenesis (Kurz et al., 2002; Lammer et al., 1998; Liakopoulos et al., 1999). SUMOylation of substrate proteins was linked to different processes including cell division, nuclear transport and transcriptional regulation (Gill, 2004), whereas the ubiquitin-like modifier ATG8 is involved in membrane dynamics during autophagy (Ichimura et al., 2000; Mizushima et al., 1998).

In contrast to ubiquitin-like modifiers, ubiquitin domain proteins (UDPs) comprise a heterogeneous group of proteins, that are characterised by the ubiquitin-like (UBL) domain. This UBL domain is an integral part of the protein, which in contrast to ubiquitin-like modifiers is neither processed nor conjugated to other proteins. Apart from their UBL domains UDPs contain additional structural elements linking them to different cellular functions (Fig. 4). However, recent studies have revealed that many UDPs share the ability to interact with the 19S regulatory particle of the 26S proteasome. This ability depends entirely on their integral UBL domain and was considered to be a general feature of all UDPs (Hartmann-Petersen and Gordon, 2004).

1.2.2 UDPs as substrate carriers for the proteasome

The best characterised UDPs are the *Saccharomyces cerevisiae* proteins Rad23p and Dsk2p, whose human orthologues are HHR23A and HHR23B and ubiquilin-1 to -4, respectively (Fig. 4). Rad23p was originally found to be involved in nucleotide excision repair of UV-damaged DNA (Watkins et al., 1993), whereas Dsk2p was implicated in spindle pole duplication (Biggins et al., 1996).

In 1998, Schauber and co-workers demonstrated that the UBL domains of yeast Rad23p and its human homologue HHR23B interact with the proteasome (Schauber et al., 1998). This finding led to the proposal that UBL domains represent a novel class of proteasome-interacting motifs and that other UDPs might also interact with the proteasome.

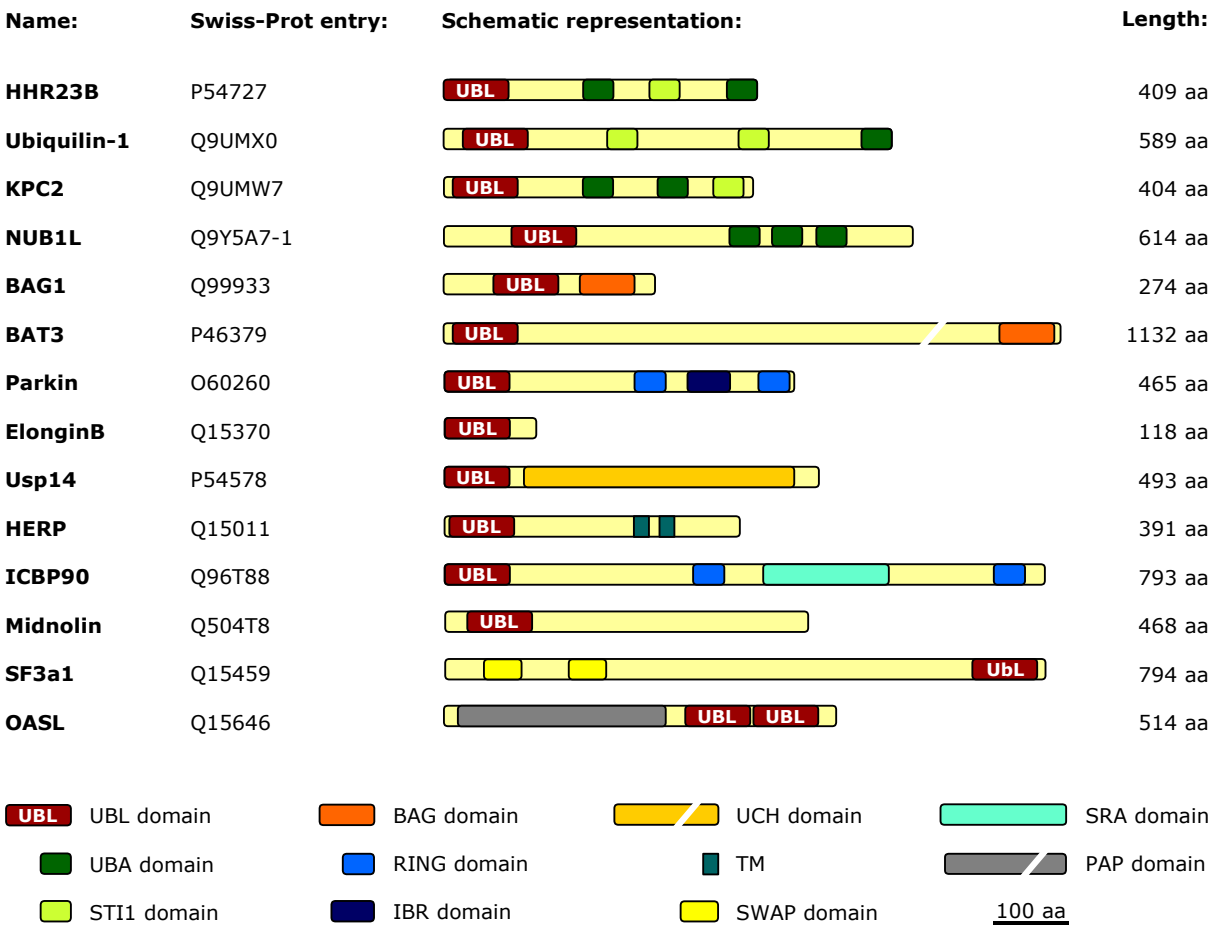

Figure 4. Domain architecture of selected human UDPs. Schematic representation of the domain architecture of selected human UDPs. The various domains are color-coded according to the key below. Details are described in the text.

In fact, the UBL domains of Dsk2p and its human homologues ubiquilin-1 and ubiquilin-2 were also found to bind the proteasome via their UBL domains (Funakoshi et al., 2002; Kleijnen et al., 2000). In addition, certain other UDPs were shown to interact with the proteasome in a UBL-dependent manner. Thus, the UBL domain was considered a general interaction motif for the proteasome.

Downstream of their N-terminal UBL domain Rad23p and Dsk2p as well as their orthologues contain one or more ubiquitin-associated (UBA) domains (Fig 4). The UBA domain functions as a ubiquitin binding domain (Hofmann and Bucher, 1996; Rao and Sastry, 2002; Wilkinson et al., 2001). In contrast to other ubiquitin interacting modules the UBA domain was shown to have a significantly higher affinity for polyubiquitin chains than for monoubiquitin (Bertolaet et al., 2001; Wilkinson et al., 2001). Since only polyubiquitylated substrates are

degraded by the 26S proteasome (Thrower et al., 2000), such UBL/UBA proteins might have a function in protein degradation. Their ability to interact with the proteasome via the UBL domain while they use their UBA domain to bind polyubiquitylated proteins hints that they act as adaptors recognising ubiquitin conjugates and shuttling them to the proteasome for their degradation (Hartmann-Petersen et al., 2003). First evidence in support of this hypothesis came from experiments in fission yeast showing that ubiquitin conjugates and the ubiquitin-proteasome substrate Rum1 are stabilised in an *S. pombe* double mutant, in which the Rad23p and Rpn10p homologues were deleted. Single mutants of either one of these proteins had no stabilising effect on Rum1 (Wilkinson et al., 2001). In budding yeast Rad23p and Dsk2p were also shown to be required for the degradation of ubiquitin-fusion substrates (Chen and Madura, 2002; Rao and Sastry, 2002). Another study demonstrates that the human Dsk2p homologues ubiquilin-1 and ubiquilin-2 associate with the proteasome, but also with E3 ligases such as E6AP as well as with their substrates (Kleijnen et al., 2000). Later, several groups took advantage of cell free systems to investigate the function of UBL/UBA proteins as recruitment factors for proteasomal substrates (Raasi and Pickart, 2003; Verma et al., 2004). Verma and co-workers isolated 26S proteasomes from *rpn10*-null and *rad23*-null yeast strains, which both displayed a deficiency in the deubiquity-lation and degradation of the proteasome substrate Sic1. These effects could be rescued by addition of either recombinant Rpn10p or Rad23p. Since Rad23p could also compensate for the Rpn10p phenotype in this study these proteins appear to have overlapping functions in shuttling ubiquitin conjugates to the proteasome for their degradation (Verma et al., 2004).

The human orthologues of Rad23p and Dsk2p, HHR23A and HHR23B as well as ubiquilin-1, -2 and -4 were also demonstrated to bind to the proteasome (Hiyama et al., 1999; Kleijnen et al., 2000; Riley et al., 2004; Schauber et al., 1998). Apart from these UDPs the human genome encodes other proteins with both a UBL and a UBA domain. KPC2 (Fig. 4), for instance, is required for the efficient degradation of p27. KPC2 was first identified in a complex with the E3 ligase KPC1. While KPC1 ubiquitylates p27, KPC2 shuttles ubiquitylated p27 to the proteasome for degradation (Hara et al., 2005; Kamura et al., 2004). Another example of a human UBL/UBA protein is NUB1 (Fig. 4), which is responsible for the recruitment of the ubiquitin-like modifiers NEDD8 and FAT10 to the proteasome for their degradation (Hipp et al., 2005; Hipp et al., 2004; Kamitani et al., 2001; Kito et al., 2001).

1.2.3 UDPs as ubiquitin-protein ligases

Apart from their function in substrate recruitment to the 26S proteasome UDPs have also been linked to other processes within the ubiquitin-proteasome system. Some UDPs, for instance, display ubiquitin-protein ligase activity. The RING E3 parkin is undoubtedly the best studied representative of this group. It contains an N-terminal UBL domain and two E3-type RING finger motifs separated by an in-between-RING (IBR) domain (Fig. 4). Interestingly, mutations in the gene encoding parkin were found to be responsible for most cases of a rare neurodevelopmental disorder called autosomal-recessive juvenile parkinsonism (Kitada et al., 1998). This condition in many ways resembles Parkinson´s disease. Thus, the corresponding gene was named *parkin*. Most of the disease-causing mutations affect the C-terminus of parkin and impair its E3 ligase activity (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). By implication, loss of parkin function may result in the accumulation of its substrate proteins, which then leads to the death of dopaminergic neurons in the substantia nigra (Imai et al., 2001; Shimura et al., 2001). A few mutations have also been identified in the UBL domain-encoding region of *parkin*. NMR studies suggested an interaction between parkin´s UBL domain and the proteasome subunit S5a/Rpn10p, which is disrupted in the pathogenic parkin mutant R42P (Sakata et al., 2003).

Another UDP is ElonginB, a component of a multisubunit E3 ligase called the CBC^{VHL} (Cullin-ElonginBC-VHL) complex. Similar to the SCF complex, it also contains the catalytic subunit RBX1. However, substrate recognition is mediated by VHL instead of an F-box protein and the two adapter proteins ElonginB and ElonginC link VHL to the Cullin (Kaelin, 2002). Since ElonginB contains a UBL domain it might tether the CBC^{VHL} complex to the proteasome to guarantee the efficient degradation of its substrates, but this interaction has not been demonstrated so far.

1.2.4 Other functions of UDPs within the ubiquitin-proteasome system

The co-chaperone BAG-1 (Fig. 4) contains, apart from its UBL domain, a BAG domain, which interacts with the ATPase domain of the molecular chaperones Hsc70/Hsp70. This interaction induces nucleotide exchange as well as substrate release and thus negatively regulates the refolding events of the molecular chaperones (Hohfeld and Jentsch, 1997; Sondermann et al., 2001; Takayama et al., 1997). Since BAG-1 also binds the proteasome via its UBL domain (Luders et al., 2000), it is likely that its function is to trigger the release of unfolded proteins from Hsc70/Hsp70, subjecting them to proteasome-dependent degradation. Indeed, this hypothesis

was supported by the finding that BAG-1 forms a ternary complex with Hsc70/Hsp70 and the ubiquitin-protein ligase CHIP and promotes CHIP-dependent degradation of the glucocorticoid hormone receptor (Demand et al., 2001).

USP14 and its yeast homologue Ubp6p are deubiquitylating enzymes that harbour UBL domains. Like the other UDPs mentioned so far, they associate with the 26S proteasome (Borodovsky et al., 2001; Chernova et al., 2003; Leggett et al., 2002). Reduced USP14 levels in a mouse model were shown to also lead to decreased levels of ubiquitin, which is consistent with results from yeast lacking Ubp6p (Anderson et al., 2005; Chernova et al., 2003). Addition of proteasome inhibitors led to a partial recovery of the cellular ubiquitin supply (Chernova et al., 2003). This suggests that in the absence of USP14/Ubp6p ubiquitin might be degraded by the proteasome along with the substrate it is attached to. These results accentuate the necessity of deubiquitylating enzymes in ubiquitin recycling to prevent proteasomal degradation of the modifier and to maintain the pool of free ubiquitin in the cell.

The subject of the study presented here is the homocysteine-inducible, ER-resident ubiquitin-like protein (HERP). Apart from its N-terminal UBL domain it contains transmembrane helices, which anchor the protein in the membrane of the endoplasmatic retikulum (ER). Interestingly, the expression of HERP is upregulated upon accumulation of misfolded proteins in the ER (Kokame et al., 2000) by the unfolded protein response pathway, which will now be introduced in further detail.

1.3 Unfolded protein response and ER-associated degradation

1.3.1 The endoplasmic reticulum and ER quality control

The endoplasmic reticulum (ER) is the site of entry into the secretory pathway. It is the cellular compartment responsible for the synthesis, folding and modification of secretory and cell-surface proteins, as well as the resident proteins of the secretory pathway. Proteins are directed to the ER by a hydrophobic signal sequence and traverse the ER membrane either co- or posttranslationally through a narrow channel formed by the heterotrimeric Sec61 complex (Matlack et al., 1998). Once in the ER, proteins fold into their native conformation and undergo a variety of posttranslational modifications such as N-linked glycosylation and the formation of disulfide bonds. These folding and modification events are conducted by an ER quality control system. An elaborate system of chaperones assists proteins in their folding and maturation and guarantees

that only properly folded proteins are exported to the Golgi compartment. Misfolded proteins are recognised and retained in the ER to complete their folding process. When large amounts of misfolded proteins accumulate in the ER, they cause ER stress and trigger an ER stress response, called the unfolded protein response (UPR). This pathway involves extensive signalling between the ER and the nucleus leading to the expression of UPR target genes to enhance the folding capacity of the ER and to reduce its unfolded protein burden. This is achieved by three mechanisms: (1) the downregulation of general protein synthesis on translational level to prevent further protein accumulation, (2) the induction of molecular chaperones and foldases that help to complete the folding process and (3) the induction of ER associated protein degradation (ERAD) for the clearance of unfolded proteins from the ER. When these adaptive responses are not sufficient to relieve ER stress, the damaged cell undergoes apoptosis (Schroder and Kaufman, 2005).

1.3.2 Recognition of unfolded proteins in the ER

The UPR is initiated whenever protein folding in the ER is compromised. Physiological conditions that induce the UPR are, for instance, altered metabolic conditions such as glucose deprivation or calcium mobilisation. However, ER-resident proteins that cannot be folded appropriately as a result of mutations are also common substrates of the UPR. The cystic fibrosis transmembrane conductance regulator (CFTR) and its common mutation ΔF508 serve as an example in this context. CFTR-ΔF508 is functional, but it folds so slowly that it is degraded via ERAD (Jensen et al., 1995; Ward et al., 1995). Proteins that lack their interaction partner also induce the UPR and are degraded via ERAD, e.g. individual subunits of the T-cell receptor like Cd3-delta or TCR-α (Tiwari and Weissman, 2001; Yang et al., 1998).

For the recognition of unfolded proteins in the ER the cell relies on factors that primarily recognise substructures within the proteins such as hydrophobic patches, unpaired cysteines and immature glycans. The mammalian lectin-type chaperones calnexin/calreticulin (CNX/CRT), for example, retain immature glycoproteins in the ER and assist in their folding (Helenius and Aebi, 2004). A well defined timing mechanism ensures that another lectin called EDEM recognises proteins that remain in the unfolded state for too long (Trombetta and Parodi, 2003); (Jakob et al., 2001). These proteins are targeted for degradation by the 26S proteasome. In addition, BIP and other molecular chaperones interact with hydrophobic surfaces of misfolded proteins in the ER (Dorner et al., 1992; Plemper et al., 1997). Oxidoreductases such as Eps1p and the redox-driven chaperone PDI, on the other hand, bind free thiols and control the formation of disulfide

bonds between correct pairs of cysteine residues. They also have a role in targeting misfolded membrane proteins for degradation (Anelli et al., 2003; Dorner et al., 1992; Plemper et al., 1997; Tsai et al., 2001; Wang and Chang, 2003).

1.3.3 The unfolded protein response

Three transmembrane proteins transduce the unfolded protein signal across the ER membrane: PERK, IRE1 and ATF6 (Rutkowski and Kaufman, 2004). These transducers are inactive under nonstressed conditions due to the association of their luminal domains with the molecular chaperone BIP. Upon ER stress BIP is competitively titrated from those luminal domains by the huge excess of unfolded proteins in the ER lumen. This results in oligomerisation and activation of IRE1 and PERK via autophosphorylation of their serine/threonine kinase domains (Bertolotti et al., 2000; Liu et al., 2003). Concomitantly, the release of BIP from ATF6 permits ATF6 transport to the Golgi compartment where it is cleaved to yield a cytosolic fragment, which functions as a transcription factor (Shen et al., 2002). Thus, due to its essential role in the activation of the three transducers PERK, IRE1 and ATF6, BIP is a key mediator of the UPR.

An immediate response to ER stress is the inhibition of protein biosynthesis mediated by the activation of PERK. Activated PERK directly phosphorylates the eukaryotic translation initiation factor eIF2α. Phosphorylated eIF2α cannot form the ternary translation initiation complex with GTP and Met-tRNA$_i^{Met}$ leading to a general attenuation of translation thereby preventing the accumulation of newly synthesised proteins in the ER (Harding et al., 2001; Harding et al., 2000; Scheuner et al., 2001). Additionally, phosphorylated eIF2α promotes the selective translation of the transcription factor ATF4 (Harding et al., 2000), which leads to the transcription of UPR target genes. Another response to ER stress is the transcriptional activation of UPR target genes by IRE1 and ATF6. Activated IRE1 functions as a site-specific endoribonuclease (RNase) which cleaves a short sequence from *XBP1* mRNA. The spliced *XBP1* mRNA encodes a transcription factor which induces the expression of many UPR target genes (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). Activated ATF6 directly works as a transcription factor, which like XBP1 binds ER stress response elements (ERSE) thereby also inducing UPR target genes (Yoshida et al., 1998). As explained above, UPR target genes code for certain chaperones and folding enzymes that assist protein folding, but also for components that are required for the disposal of misfolded proteins that fail to reach their native, functional conformation.

1.3.4 ER associated protein degradation (ERAD)

Proteins that cannot be brought into their native functional conformation or proteins that lack their interaction partners are degraded via ERAD. It was initially thought that proteolysis occurs in the lumen of the ER. However, although exceptions might exist (Schmitz and Herzog, 2004), most misfolded ER proteins appear to be translocated back to the cytoplasm and degraded by the 26S proteasome (Sommer and Jentsch, 1993; Hiller et al., 1996; Jensen et al., 1995; Ward et al., 1995). This ERAD pathway can be conceptualised as four different steps: (1) substrate recognition and targeting to the retrotranslocation machinery, (2) protein transport across the ER membrane and ubiquitylation, (3) release of the substrate from the ER membrane to the cytosol and (4) transfer of the substrate to the 26S proteasome and its degradation (Fig. 5).

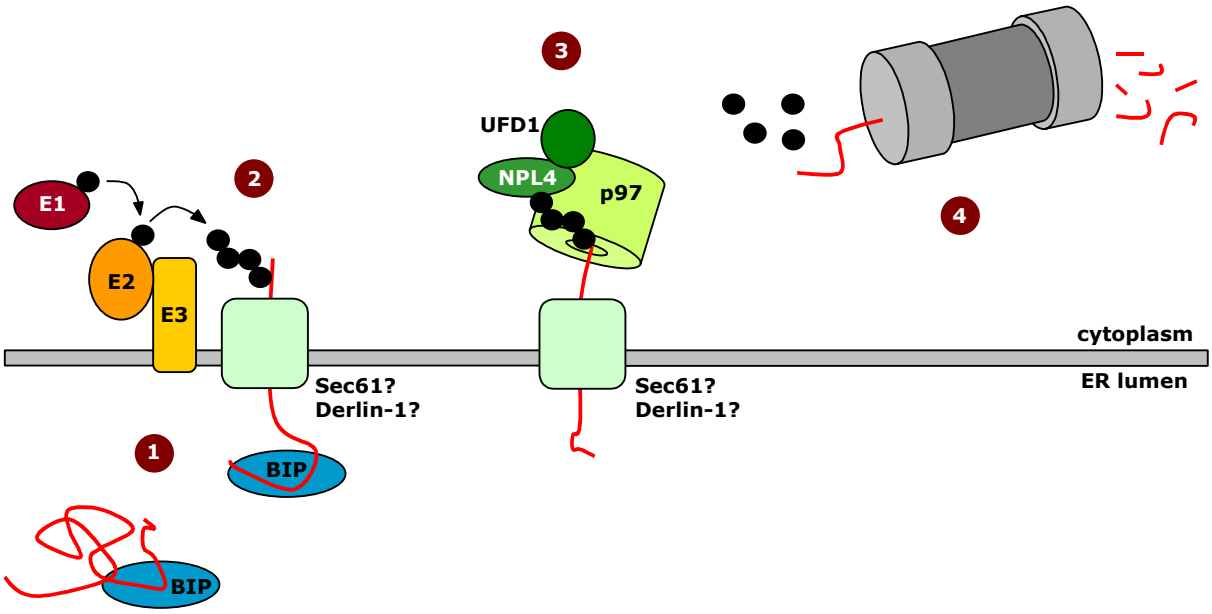

Figure 5. The ERAD pathway. ER associated protein degradation occurs in four steps: **(1)** Misfolded proteins are recognised by different ER-resident chaperones such as BIP and recruited to the retrotranslocation channel. **(2)** Proteins are transported through the channel and are polyubiquitylated at the cytoplasmic face of the ER membrane. **(3)** Polyubiquitylated proteins are recognised by the ATPase p97 and dislocation is completed by p97 and its cofactors NPL4 and UFD1. **(4)** Fully released proteins are conveyed to the the proteasome and degraded.

(1) Substrate recognition and recruitment to the retrotranslocation channel

The recognition of unfolded lumenal protein domains is mediated by ER resident molecular chaperones such as BIP and PDI. However, cytoplasmic chaperones such as HSP70 and HSP90 can also be involved in the recognition of misfolded membrane proteins, as they target misfolded

cytoplasmic domains of ER membrane proteins. HSP70 and HSP90 were, for instance, shown to target the unfolded membrane protein CFTR, which is subsequently ubiquitylated by CHIP, an E3 ligase, that is associated with cytoplasmic chaperones. (Connell et al., 2001); (Meacham et al., 2001). After their recognition unfolded proteins are directed to the retrotranslocation channel and exported.

It was also suggested that membrane proteins may be extracted from the ER by the proteasome itself (Mayer et al., 1998; Walter et al., 2001). However, most membrane proteins and all soluble proteins are thought to be retrotranslocated to the cytosol by a protein-conducting channel. Several lines of evidence suggest that this channel is made of the Sec61 complex, which is also involved in the import of proteins into the ER. (Pilon et al., 1997; Plemper et al., 1997; Plemper et al., 1998; Wiertz et al., 1996; Zhou and Schekman, 1999). However, recently this view has been challenged and it has been proposed that the transmembrane protein Derlin-1 forms the retrotranslocation pore (Lilley and Ploegh, 2004; Ye et al., 2004). Thus, the details of this retrotranslocation of substrate proteins still remains controversial to date.

(2) Protein transport and ubiquitylation

While still associated with the ER membrane dislocated proteins are ubiquitylated. In yeast the E2 enzymes Ubc6p and Ubc7p and the E3 ligases Hrd1p and Doa10p mediate the ubiquitylation of ERAD substrates (Bays et al., 2001; Hiller et al., 1996; Sommer and Jentsch, 1993; Swanson et al., 2001). Mammalian counterparts of the E2 enzymes Ubc6p and Ubc7p have been described to act as ubiquitin conjugases in ERAD as well (Kim et al., 2003; Lenk et al., 2002). Similarly, mammalian homologues of yeast E3 ligases exist. The human E3 ligases HRD1 and gp78, for instance, are orthologues of the yeast Hrd1p and have been shown to mediate the ubiquitylation of ERAD substrates such as TCR-α and Cd3-delta (Bays et al., 2001; Kikkert et al., 2004; Fang et al., 2001). Apart from HRD1 and gp78, additional ubiquitin ligases participate in mammalian ERAD. CHIP was shown to mediate the ubiquitylation of mutated CFTR (Connell et al., 2001; Meacham et al., 2001). Parkin may also play a role in ERAD by ubiquitylating membrane-bound proteins (Imai et al., 2002; Imai et al., 2001). Another example is an SCF-complex associated with the F-box proteins Fbs1 or Fbs2. These proteins recognise N-glycoproteins and thus this SCF^{Fbs} complex has been suggested to ubiquitylate dislocated glycoproteins (Yoshida et al., 2002; Yoshida et al., 2003). Interestingly, parkin and HRD1 were shown to be upregulated upon ER stress (Imai et al., 2000; Kaneko et al., 2002) accentuating their special function for the elimiation of misfolded ER proteins.

(3) Release of substrates from the ER membrane to the cytosol

In addition to acting as a degradation signal for the proteasome, polyubiquitylation is also required for retrotranslocation of substrate proteins from the ER to the cytosol (Kikkert et al., 2001). This is performed by an ATPase complex consisting of a p97 (also called VCP or, in yeast Cdc48) homohexamer and the proteins Ufd1 and Npl4. This p97-Ufd1-Npl4 complex was shown to bind ubiquitin conjugates (Dai and Li, 2001; Meyer et al., 2002; Rape et al., 2001; Wang et al., 2003) and to be required for the retrotranslocation of various substrates (Bays et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). Although the mode of action of this ATPase complex in ERAD is unclear so far, it might act as a motor that actively pulls ubiquitylated molecules out of the ER membrane (Ye et al., 2003). Binding of p97 to the ER membrane is required for this process (Ye et al., 2003). It was proposed that a membrane protein called VIMP serves as a membrane receptor for the p97 complex by linking p97 to Derlin-1, the putative retrotranslocation channel (Ye et al., 2004).

(4) Substrate recruitment to the 26S proteasome and degradation

Once the protein substrate is released into the cytosol it might be further ubiquitylated by the E4 enzyme Ufd2, which was shown to associate with the p97 homologue Cdc48p in yeast (Koegl et al., 1999). However, the exact mechanism of how substrate proteins derived from the ER are recruited to the proteasome has been unknown so far.

1.3.5 Other ERAD substrates

ERAD was initially thought to exclusively degrade misfolded and orphan secretory proteins. However, it is now clear that the ERAD pathway is also required for the regulated turnover of native ER proteins that play important roles in cellular homeostasis. In yeast and in mammalian cells, for example, the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is poly-ubiquitylated and degraded via ERAD in response to sterols. HMGR catalyses the rate limiting step in the production of sterols and its derivatives. When sterols are present, they induce the degradation of HMGR to inhibit the overproduction of sterols (Hampton et al., 1996; Ravid et al., 2000). Another example is the inositol 1,4,5-triphosphate (IP_3) receptor, which is rapidly degraded in response to stimulation of G protein-coupled receptors (Bokkala and Joseph, 1997; Oberdorf et al., 1999).

In addition, certain viruses exploit the ER quality control system to destroy key elements of the host immune defence. For example US2 and US11, two proteins of the cytomegalovirus (HCMV) bind to the newly synthesised major histocompatibility (MHC) class I heavy chain in the

ER and initiate its dislocation into the cytoplasm where it is degraded by the proteasome (Wiertz et al., 1996). Thus, ERAD has important consequences for diverse aspects of cell physiology including protein folding, metabolic regulation, signal transduction and immune response.

1.3.6 ER stress induced apoptosis

If the overload of unfolded proteins in the ER is not resolved by refolding processes or ERAD, prolonged UPR activation leads to programmed cell death. ER stress induced apoptosis can also be induced by agents, which inhibit ER function, such as tunicamycin, a specific inhibitor of N-glycosylation, thapsigargin, which inhibits the Ca^{+}-ATPase channel, or the reducing component β-mercaptoethanol. These agents cause ER stress leading to apoptosis since the adaptive responces are not sufficient to diminish ER stress (Kim et al., 2006).

Three major proteins control ER stress induced apoptosis: IRE1, caspase-12 and CHOP. In response to ER stress IRE1 can trigger a signalling cascade, which eventually leads to mitochondria/Apaf-1-dependent caspase activation (Leppa and Bohmann, 1999; Nishitoh et al., 2002; Urano et al., 2000). This results in caspase-9 activation, which in turn activates caspase-3, leading to apoptosis. However, upon ER stress induced apoptosis caspase-9 can also be activated independently of Apaf-1 (Rao et al., 2002). Caspase-12 is an ER-associated proximal effector of the caspase activation cascade. It is synthesised as an inactive proenzyme, which is activated in response to ER stress, not however, following other death stimuli (Nakagawa et al., 2000; Szegezdi et al., 2003). Activated caspase-12 initiates a caspase cascade through cleavage of procaspase-9 and -3 leading to apoptosis (Rao et al., 2002). Finally, ER stress also mediates the transcriptional activation of genes encoding proapoptotic factors. *CHOP* expression, for instance, is induced by the PERK/ATF4 and ATF6 pathways (Ma et al., 2002). CHOP, which is also known as GADD153, is a transcription factor, that potentiates apoptosis by the downregulation of the apoptosis repressor Bcl-2 (McCullough et al., 2001).

1.3.7 The UDP HERP and the unfolded protein response

Recently, a novel protein was described by two groups to be upregulated by the UPR pathway. Interestingly, this protein contains a UBL domain. Van Laar and co-workers showed that apart from ER stress the gene is also induced in response to osmotic stress and DNA damaging agents such as methyl methanesulfonate (MMS). Accordingly, it was named Mif1 for MSS inducible factor. However, ER stress inducing agents such as tunicamycin appeared to be the strongest

enhancer of *Mif1* expression. The *Mif1* promoter contains ERSE-like sequences that are binding sites for the UPR dependent transcription factors ATF6 and XBP1 (van Laar et al., 2000). Kokame and co-workers studied ER stress and showed that the mRNA and protein levels of the novel protein were enhanced by various ER stress inducing agents such as homocysteine. Since the protein appeared to localise to the ER they decided to name it HERP for homocysteine-inducible ER-resident ubiquitin-like protein.

Biochemical experiments revealed that HERP is an integral membrane protein with both termini facing the cytoplasm. Based on a hydrophobicity plot the authors proposed two models (Fig. 6) for the membrane topology of HERP (Kokame et al., 2000). The UBL domain indicated a role of HERP in the ubiquitin-proteasome system. As its synthesis is induced upon ER stress it might have a specific role in the ERAD pathway, possibly by recruiting the proteasome to the site of ERAD. However, its function within the URP has been unknown so far.

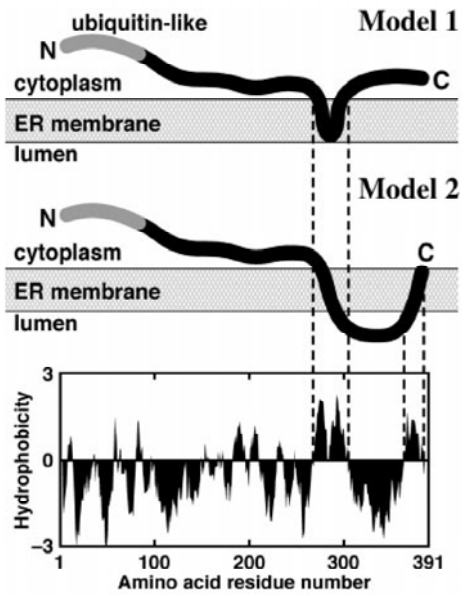

Figure 6. Membrane topology of HERP. HERP is a mammalian UDP containing an N-terminal UBL domain. It is anchored in the ER membrane with the majority of the protein facing the cytoplasm. The hydropathic profile of the amino acid sequence of human HERP was obtained using the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982). Positive values represent transmembrane helices (from Kokame et al., 2000).

1.4 Aim of this study

The aim of this study was the structural and functional characterisisation of the HERP protein. Since HERP was shown to be upregulated by the UPR, this suggested a role either as a chaperone to assist in protein folding within the ER or as an ERAD component to eliminate accumulated proteins from the ER. However, the following points led to the speculation that HERP has a role in ERAD rather than functioning as a chaperone:

(1) The majority of the HERP protein is localised to the cytoplasmic side of the ER, where certain steps of the ERAD pathway occur. (2) HERP contains a UBL domain, which suggests a role for HERP in the ubiquitin-proteasome system. Since many UDPs were shown to interact with the proteasome its role might be to recruit the 26S proteasome to the ER for efficient ERAD. (3) Many UDPs were shown to bind E3 ligases or even display E3 ligase activity themselves. (4) Northern blot analyses from *HERP* mRNA and mRNA coding for the ERAD E3 ligase HRD1 revealed a strikingly similar distribution of both transcripts in many human organs (Kaneko et al., 2002; Kokame et al., 2000). And (5) both proteins HERP and HRD1 are integral transmembrane proteins, which localise to the ER and are upregulated by the UPR. This indicates that HERP might act in close cooperation with the E3 ligase HRD1 during ERAD. Therefore, this study focuses on investigating the role of HERP within the UPR to test the hypothesis that HERP has a function in human ERAD.

2 MATERIALS AND METHODS

2.1 Molecular biology

2.1.1 Cultivation and storage of *Escherichia coli*

Liquid cultures of *E. coli* were generally grown in LB media (1% bacto trypton, 0.5% yeast extract, 1% NaCl for agar plates add 1.5% agar) shaking at 200 rpm at 37°C, except when dealing with protein expression which was mediated at room temperature (RT). Cultures on agar plates were incubated at 37°C. For the selection of transformed bacteria 100 μg/mL ampicillin or 50 μg/mL kanamycin were added to the medium. The culture density was determined by measuring the absorbance at a wavelength of 600 nm (OD_{600}). For long-term storages, cultures in stationary phase were frozen at -80°C in LB medium containing 15% (v/v) glycerol.

2.1.2 Isolation of plasmid DNA from *E. coli* cells

"Mini-Prep" with alkaline lysis

To screen for positive clones derived from a transformation, 5 mL LB medium containing the appropriate antibiotic were inoculated with a single *E. coli* colony and shaken over night at 37°C. One mL of the resulting culture was centrifuged in an eppendorf tube at 10,000x g for 5 min to spin down the cells. The pellet was resuspended in 300 μL buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 μg/mL RNaseA). Then, 300 μL buffer P2 (200 mM NaOH, 1% SDS w/v) were added and the suspension was mixed by inverting the tube. After the addition of 300 μL buffer P3 (3 M potassium acetate, pH 5.5) and mixing the suspension was centrifuged for 10 min at 13,000x g. The supernatant was transferred into a new tube and the DNA was precipitated with 500 μL isopropanol. After another centrifugation for 20 min at 13,000x g the DNA pellet was washed with 70% ethanol, dried at RT and resuspended in 50 μL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

"Mini-Prep" via affinity purification

Plasmid DNA for transformations or sequencing reactions was isolated with the GFX Micro Plasmid Prep Kit (Amersham). This kit yields up to 40 μg/mL plasmid DNA from *E. coli* cultures. The isolation was performed according to the manufacturer´s instructions.

Isolation of large amounts of plasmid DNA

Transfection of mammalian cells required large amounts of plasmid DNA. These were prepared using the Plasmid Midi and Maxi kits (Qiagen) and performed as described in the manufacturer´s instructions.

2.1.3 Separation of DNA fragments by gel electrophoresis

DNA restriction fragments or cDNA fragments were electrophoretically separated on 1-2% agarose gels containing 0.1 μg/mL ethidium bromide (Sambrook, 1989). Agarose was diluted in 1x TAE buffer, which was also used as running buffer. DNA samples were mixed with 10x DNA loading buffer and electrophoretically separated at 10 V/cm. DNA fragments were visualised by the intercalation of ethidium bromide into the DNA using a UV transluminator. The size of the fragments was estimated by standard size markers such as the 100 bp ladder (MBBL) or the 1 kb ladder (Invitrogen).

50x TAE buffer (pH 8.0): 2 M Tris, 1 M acetic acid, 50 mM EDTA
10x DNA loading buffer: 40% sucrose, 0.5% SDS, 0.25% bromophenol blue (sodium salt) in TE buffer (pH 8.0)

2.1.4 Isolation of total RNA from HeLa cells

Total RNA from HeLa cells was utilised for RT-PCR analysis and for the preparation of cDNA used for cloning procedures. For RT-PCR analysis HeLa cells were left untreated or were treated with 10 mM β-mercaptoethanol for 4 h or with 200 U/mL interferon-γ for 24 h. HeLa cells (5 x 10^6) on a 10 cm culture dish were washed with PBS and lysed directly on the culture dish with 1 mL Trizol™ (Invitrogen). Cells were scraped off the dish with a cell scraper and transferred into an eppendorf tube. After the suspension was incubated at RT for 5 min the tube was centrifuged at 13,000x g for 5 min and the cell debris was disposed of. Then, 200 μL chloroform were added to the supernatant. The suspension was mixed thoroughly, incubated at RT for 10 min and centrifuged at 13,000x g and 4°C for 10 min. The resulting aqueous upper phase, containing the RNA, was transferred into a new tube. Addition of 500 μL isopropanol resulted in precipitation of the RNA. After 10 min incubation at RT the RNA was pelleted by centrifugation, washed with 70% ethanol and diluted in 20 to 50 μL RNase free water. The quality of RNA was checked by agarose gel electrophoresis with RNA loading buffer (see section 2.1.6). Two sharp bands corresponding to 28S and 18S rRNAs and little to no background were observed.

2.1.5 Determination of DNA and RNA concentration in solution

DNA and RNA concentrations were photometrically determined with a Genesys 10^{TM} UV spectrophotometer (Thermo Electron) by measuring the absorbance at a wavelength of 260 nm (Sambrook, 1989). An OD_{260} of one equals a concentration of 50 μg/mL double stranded DNA, 40 μg/mL single stranded DNA and RNA or 33 μg/mL oligonucleotides.

2.1.6 Denaturing agarose gel electrophoresis of RNA

Total RNA from different human organs (BD Biosciences, Clontech) was electrophoretically separated in denaturing agarose gels, which were prepared as follows. 1.5 g agarose was diluted in 10 mL 10x MOPS buffer (pH 7.0) and 73 mL RNase-free water and the mixture was boiled to dissolve the agarose. After the agarose solution was cooled down to 60°C, 17 mL of a 37% formaldehyde solution were added and the gel was cast. The polymerised gel was placed in a tank and 1x MOPS (pH 7.0) running buffer was added to cover it. The RNA samples were prepared by adding 0.25 volumes 5x RNA loading buffer, followed by an incubation at 68°C for 10 min. The RNA samples (2 μg each) and a standard size marker RNAI (Roche) were loaded onto the gel and electrophoresis was performed at 5-6 V/cm. RNA bands were then visualised in a UV transilluminator.

5x RNA loading buffer: 200 μL 1% bromophenol blue, 80 μL 500 mM EDTA, 100 μL ethidium bromide (10 mg/mL), 720 μL 37% (= 12.3 M) formaldehyde, 2 mL 100% glycerol (2.52 g), 3084 μL formamide, 4 mL 10x MOPS, add DEPC-H_2O to 10 mL

10x MOPS buffer (pH 7.0): 200 mM MOPS, 500 mM sodium acetate, 10 mM EDTA

2.1.7 Northern blotting

After electrophoresis a photograph was taken of the gel. It was then rinsed briefly with RNase-free water, shaken in RNase-free water for 15 min, in 50 mM NaOH for 30 min and then in 20x SSC (3 M NaCl, 0.3 M sodium acetate) for 15 min. RNA transfer onto a positively charged nylon membrane (Roche) was performed by capillary action over night in 20x SSC. To do so, the gel was put onto two Whatman 3MM papers, which had contact to a 20x SSC reservoir. The nylon membrane was first soaked in water, then in 20x SSC and put on top of the gel. Then, two Whatman 3 MM papers soaked in 20x SSC and a stack of dry paper were put onto the membrane and the stack was compressed with a weight. After transfer, the nylon membrane was rinsed with 2x SSC and treated with UV-light for 3 min to crosslink the RNA to the membrane.

2.1.8 DIG labelling of RNA probes

Generation of DIG labelled RNA probes for hybridisation of Northern Blots was performed with the Random Primed DIG RNA Labelling Kit (Roche). First, the gene of interest was amplified to generate PCR products that contained an RNA polymerase promotor site at the 5´ end. This was done with primers, which amplified about 1 kb of the target sequence. The antisense primer contained a T7 RNA polymerase promoter (TAATACGACTCACTATAGGG) at the 3´ end. Subsequently, the PCR product was purified via agarose gel electrophoresis. For the labelling reaction 5 µL of the PCR product (100-200 ng), 2 µL DIG RNA 10x labelling mix, 2 µL transcription buffer and 2 µL T7 RNA polymerase were mixed and sterile deionised water was added to a final volume of 20 µL. The sample was incubated at 37°C for 2 h. The polymerisation reaction was stopped by addition of 2 µL 0.2 M EDTA, pH 8.0. The RNA was precipitated with 2.5 µL LiCl and 75 µL ethanol for 1 h at –70°C or over night at –20°C and centrifuged for 30 min at 13,000x g and 4°C. Subsequently, the RNA pellets were washed with 50 µL 70% ethanol, dried and resuspended in 100 µL sterile RNase-free water. The efficiency of the labelling reaction was determined semiquantitatively by a dilution series of the DIG labelled RNA. To do so, 1 µL of DIG labelled RNA was pipetted onto a positively charged nylon membrane in a dilution series from 10^{-2} to 10^{-7}, crosslinked with UV-light and incubated with the DIG-specific antibody as described below. The labelling reaction was efficient when the 10^{-5} dilution gave a signal with the DIG-specific antibody.

2.1.9 DIG hybridisation of Northern Blots

The nylon membrane with the blotted total RNA was incubated in 20 mL hybridisation solution for 2 h at 68°C. Then, 5-10 µL of DIG labelled RNA probe were added to the hybridisation solution and incubated over night at 68°C. On the next day the Northern blot was washed twice for 5 min with 2x SSC, 0.1% SDS and twice for 15 min with 0.2x SSC, 0.1% SDS at 68°C. The membrane was equilibrated in 1x maleic acid solution at RT followed by an incubation in 1x blocking solution (10% blocking reagent (Roche) in 1x maleic acid solution) for 30 min. Then, the membrane was incubated with 1 µL of anti-DIG antibody (Roche) in 1x blocking solution for 30 min. Afterwards, the membrane was washed twice for 15 min with 1x maleic acid buffer, equilibrated for 3 min in detection buffer (100 mM Tris pH 9.5, 100 mM NaCl) and incubated for 5 min in 2 mL CDP-Star (Roche). The signals were visualised by exposing the membrane to a Biomax-MR film (Kodak) for 1 to 15 min depending on the signal intensity.

Hybridisation solution: 5x SSC, 0.1% N-lauroylsarcosin (sodium-salt, Sigma), 7% SDS, 1% blocking reagent (Roche), ad 500 mL with RNase-free H_2O

10x maleic acid solution: 1 M maleic acid, 1.5 M NaCl, ad 1 L, adjust pH to 7.5 with NaOH

Table 1. Oligonucleotides for Northern blot and RT-PCR analysis. Fw indicates forward, rev reverse primers used in this study.

Name	Sequence (5´ - 3´)	Comment
AS13	GCTGCTTCTTGAACTGGACC	fw-primer for DIG-labelling of MUBL2 mRNA
AS14	TAATACGACTCACTATAGGGAAGTTGTTTGC ATTTTGCCC	rev-primer for DIG-labelling of MUBL2 mRNA
AS11	CTGGGAAGCTGTTGTTGGAT	fw-primer for HERP 337 bp fragment, RT-PCR
AS29	GAAAGCTGAAGCCACCCATA	rev-primer for HERP 337 bp fragment, RT-PCR
AS13	GCTGCTTCTTGAACTGGACC	fw-primer for MUBL2 354 bp fragment, RT-PCR
AS30	TACGCTGCCTCAATCCTTCT	rev-primer for MUBL2 354 bp fragment, RT-PCR
AS15	GGGTAATGAGGTGATGGTGG	fw-primer for MUBL3 356 bp fragment, RT-PCR
AS31	CTGCACCTGCTTGTCTTTTG	rev-primer for MUBL3 356 bp fragment, RT-PCR
AS17	ATGACCCTGATTGAAGGGGT	fw-primer for MUBL4 712 bp fragment, RT-PCR
AS31	TCCTTTTCAAGGAGCCAATG	rev-primer for MUBL4 712 bp fragment, RT-PCR
Actin Sonde F	GGCCGGCTTCGCGGGCGACG	fw-primer for actin 1.2 kb fragment, RT-PCR
Actin Sonde R	TAATACGACTCACTATAGGGGCCGGACTCGT CATACTC	rev-primer for actin 1.2 kb fragment, RT-PCR
Oligo (dT)	TTTTTTTTTTTTTTTTT	for reverse transcription

2.1.10 RT-PCR

cDNA was synthesised from RNA via a reverse transcriptase reaction. This was done using poly-dT primers which bound to the polyadenylated mRNAs. 2 µg total RNA and 1 µL poly-dT primers were denatured for 5 min at 70°C. Then, 5 µL 5x reverse transcriptase buffer, 0.5 µL RNase inhibitor (40 U/µL), 0.5 µL dNTP mix (100 mM) and 1 µL M-MLV reverse transcriptase (Promega) were added and the solution was topped up to 10 µL with water. The reaction mixture was incubated for 1 h at 42°C, cooled to 4°C and denatured at 95°C for 2 min. The cDNAs were either stored at -20°C or utilised directly for the PCR reaction as a template. Parallel amplification of β-actin with the sequences of interest (table 1) in a multiplex PCR permitted to do a semi-quantative analysis of the original amount of transcript in the RNA sample, which is based on the fact that β-actin mRNA is equally abundant in the samples. The PCR products were monitored by agarose gel electrophoresis.

2.1.11 Polymerase chain reaction

The PCR protocol described below was used for both the cloning of plasmid constructs and for RT-PCR. PCR reactions were performed in a volume of 50 µL with 10 ng of plasmid DNA, 20 pmol/µL of the forward and the reverse primers, 1 µL dNTP mix (NEB, each 10 mM), 3-4 U DNA polymerase (Pfu polymerase (Promega), Taq polymerase (NEB) or High-Fidelity polymerase (Roche)) in the corresponding PCR buffer supplied by the manufacturer. Amplification reactions were carried out in a PCR mastercycler (Eppendorf) or a thermocycler (Biometra). The reaction profile was adjusted according to the quantity and quality of template DNA, the length and G/C content of the oligonucleotides and the length of the amplified sequence (table 2).

Table 2. PCR program used for amplification of cDNA.

PCR program:	termperature:	time:	amount of cycles:
initial denaturation	95°C	5 min	1 x
denaturation	95°C	1 min	30 x
hybridisation	50-70°C	1 min	
elongation	72°C	1-3 min	
terminal elongation	72°C	10 min	1 x

2.1.12 *In vitro* recombination of DNA

Restriction digestion of DNA

The restriction digestion of DNA was performed with endonucleases from NEB and Fermentas according to the manufacturer´s instructions. Usually, 1 µg of DNA was incubated with 1 U enzyme in the appropriate buffer for at least 1.5 h at 37°C. The digested DNA was inspected by agarose gel electrophoresis (2.1.3).

Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from a restriction reaction, the digested DNA was separated in a 1% agarose gel containing ethidium bromide. After electrophoresis the DNA was visualised with a UV transilluminator and the fragments of interest were isolated by excising the respective piece of agarose. The DNA fragment was extracted from the agarose block using the GFX Extraction Kit (Amersham) and eluted in deionised water.

Dephosphorylation of linearised plasmid DNA

To avoid recirculation of linearised plasmid DNA the 5´ end of the vector DNA was dephosphorylated after restriction digestion and isolation from agarose gels. To do so, plasmid

DNA was diluted in a reaction buffer and 1 U shrimp alkaline phosphatase (Roche) was added. The reaction was carried out at 37°C for 1 h, followed by heat inactivation of the enzyme at 70°C for 20 min.

Generation of double-stranded DNA from oligonucleotides

For the generation of pSuper constructs containing HERP-specific sequences 1 µL of each sense and antisense oligonucleotide (10 µg/µL) were diluted in 18 µL annealing buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl), heated in a PCR machine to 95°C and then cooled down to 70°C in steps of 4°C for 4 min each. Subsequently, the sample was conveyed to a 70°C waterbath which was cooled down slowly to RT. The annealed oligonucleotides were utilised for the phosphorylation reaction.

Phosphorylation of insert DNA fragments

Before cloning, the annealed double-stranded oligonucleotides were phosphorylated at the 5´ ends with polynucleotide kinase (Roche). Seven µL of oligonucleotide solution were mixed with 1 µL ATP (10 mM), 10x reaction buffer (Roche) and 1 µL polynucleotide kinase and the sample was incubated for 1 h at 37°C. The reaction was stopped at 65°C for 10 min and without further purification utilised for the ligation reaction.

Ligation of linearised vector and insert DNA fragments

Isolated DNA fragments (inserts) and linearised vectors were run on agarose gels (2.1.3) to inspect the quality and to estimate the concentration of DNA in solution. For the ligation reaction vector and insert were mixed in ratios of 1:3 to 1:10. The reaction sample (20 µL) contained 100 ng of vector DNA and 10 U T4 DNA ligase (NEB). The reaction was performed either at RT for 2 h or at 16°C for 14 h.

Site-directed mutagenesis

For the insertion of site specific mutations into double-stranded plasmids the QuickChange site-directed mutagenesis kit was used (Stratagene). This method utilises two complementary oligo-nucleotide primers which carry the codon to be mutated in the middle flanked by 15 additional base pairs on each side that correspond to the target sequence. The procedure was carried out according to the manufacturer´s instructions. Briefly, 50 ng of template DNA were mixed with 62.5 ng of each primer, 1 µL of desoxynucleotide mix, 5 µL of 5x reaction buffer and 1 µL Pfu turbo DNA polymerase in a volume of 50 µL. To eliminate the parental plasmid DNA the PCR reaction was subsequently digested with *Dpn*I at 37°C for 1 h. After digestion the PCR product was directly used for transformation into *E. coli* XL1 blue cells provided with the kit.

2.1.13 Transformation of *E. coli* cells with plasmid DNA

For transformation of plasmid DNA after *in vitro* recombination the *E. coli* strain XL1 blue was used, whereas the *E. coli* strains BL21 De3 and M15 were used for expression of GST-tagged or His-tagged proteins, respectively. Preparation of competent cells and transformation of these *E. coli* strains was performed as described below.

Preparation of competent *E. coli* cells

E. coli cells from a glycerol stock were streaked out on an LB agar plate and incubated over night at 37°C. On the next day 5 mL of φa-medium (0.5% yeast extract, 2% bacto tryptone, 40 mM $MgSO_4$, pH 7.6) were inoculated with a single *E. coli* colony and shaken over night at 37°C. This culture was diluted 1:50 the next day and grown to an OD_{600} of 0.5. After chilling the culture on ice for 5 min the cells were transferred into sterile Falcon tubes and harvested by centrifugation (5 min, 5,000x g, 4°C). All following steps were performed with prechilled sterile materials and solutions at 4°C. The sedimented cells were resuspended in 40 mL chilled TFB-I solution and incubated on ice for at least 15 min. Then, the cells were pelleted again by centrifugation and resuspended in 4 mL chilled TFB-II solution. After 30 min on ice the cells were transferred into eppendorf tubes in 100 µL aliquots and quickly frozen either on dry ice or in liquid nitrogen and stored at -80°C.

TFB-I: 30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH adjusted to 5.8 with 0.2 N acetic acid, sterile filtered, stored at 4°C.

TFB-II: 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH adjusted to 7.0 with 1 M KOH, sterile filtered, stored at 4°C.

Transformation of *E. coli* cells

For transformation of *E. coli* 100 µL of competent cells were thawed on ice and subsequently incubated with a maximum of 50 ng plasmid DNA on ice. After a brief heat shock (42°C, 45-90 s) the cells were kept on ice for 10 min, followed by addition of 1 mL SOC medium without antibiotics and shaking at 37°C for 1 h. This suspension was streaked out in different dilutions onto agar plates containing the respective antibiotic(s) and incubated over night at 37°C to select for transformed cells.

SOC medium: 2% bacto trypton, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.0 mM glucose

2.1.14 Oligonucleotides and expression vectors

Table 3. Oligonucleotides used for expression vector construction. Fw indicates forward, rev reverse primers used in this study.

Name	Sequence (5´ - 3´)	Comment
AS21	CGCGAATTCGCCACCATGGAGTCCGAGACCGAACC	fw-primer for M134
AS22	CGCGAATTCGCCACCATGGACCAAAGTGGGATGGAGATT	fw-primer for M136
AS23	CGCGAATTCGCCACCATGGAGCTCTCTGATGTCAC	fw-primer for M138
AS24	CGCGAATTCGCCACCATGACCCTGATTGAAGGGGTGG	fw-primer for M139
AS25	CGCGGATCCGTTTGCGATGGCTGGGGGGCC	rev-primer for M134
AS26	CGCGGATCCATTGGCAACCTGGGGAGGCCC	rev-primer for M136
AS27	CGCGGATCCTCGTCCATACATCCCAAATAC	rev-primer for M138
AS28	CGCGGATCCCGGGCGGTACATGGCAAAGGC	rev-primer for M139
AS37	GATCCCCGCAGAAGAACGGCATCAAGTTCAAGAGACTTGATGCCGTTCT TCTGCTTTTTGGAAA	fw-primer for M175
AS38	AGCTTTTCCAAAAAGCAGAAGAACGGCATCAAGTCTCTTGAACTTGATGC CGTTCTTCTGCGGG	rev-primer for M175
AS41	GATCCCCGGTGGCTGAATCCACAGAGTTCAAGAGACTCTGTGGATTCAG CCACCTTTTTGGAAA	fw-primer for M207
AS42	AGCTTTTCCAAAAAGGTGGCTGAATCCACAGAGTCTCTTGAACTCTGTGG ATTCAGCCACCGGG	rev-primer for M207
AS45	GATCCCCGCTGTTGTTGGATCACCAATTCAAGAGATTGGTGATCCAACAA CAGCTTTTTGGAAA	fw-primer for M307
AS46	AGCTTTTCCAAAAAGCTGTTGTTGGATCACCAATCTCTTGAATTGGTGAT CCAACAACAGCGGG	rev-primer for M307
AS49	GATCCCCATGATGGTCCTCCTCCTGATTCAAGAGATCAGGAGGAGGACC ATCATTTTTTGGAAA	fw-primer for M309
AS50	AGCTTTTCCAAAAAATGATGGTCCTCCTCCTGATCTCTTGAATCAGGAGG AGGACCATCATGGG	rev-primer for M309

Table 4. Expression vectors for *E. coli*. MCS sites indicate multiple cloning sites used for ligation of insert and vector. pGEX-KG (Guan and Dixon, 1991), pQE30 (Qiagen), pRSET (Invitrogen).

Construct	Insert	Vector	Tag	MCS sites	comment
M5	-	pGEX-KG	GST		
M99	HERP-ΔC	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M147	HERP-UBL	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M148	HERP-ΔC-ΔUBL	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M236	HERP-ΔC-hybrid	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M237	HHR23B	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M360	HRD1-Ccr	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M363	Derlin-1-Ccr	pGEX-KG	GST	*Bam*HI/*Eco*RI	M. Seeger
M265	HRD1-Ccr	pQE30	His	*Bam*HI/*Hind*III	M. Seeger
M330	p97	pQE30	His		R. Hartmann-Petersen
M183	S5a	pQE30	His	*Bgl*II/*Bam*HI	M. Seeger
M400	USP7 N-terminus	pRSET.B	His	*Bam*HI/*Eco*RI	M. Seeger

Table 5. Expression vecotrs for mammalian cells. MCS sites indicate multiple cloning sites used for ligation of insert and vector. pEGFP (Clontech), pSG5 (Stratagene), pCDNA3.1 hygro+ (Invitrogen), pSuper (Oligoengine).

Construct	Insert	Vector	Tag	MCS sites	Comment
M134	HERP	pEGFP-N3	EGFP	EcoRI/BamHI	AS21/AS25
M136	MUBL2	pEGFP-N3	EGFP	EcoRI/BamHI	AS22/AS26
M138	MUBL3	pEGFP-N3	EGFP	EcoRI/BamHI	AS23/AS27
M139	MUBL4	pEGFP-N3	EGFP	EcoRI/BamHI	AS24/AS28
M31	ZZ	pSG5	-	BamHI	M. Seeger
M127	HERP	pSG5-ZZ (M31)	ZZ	EcoRI/BamHI	M. Seeger
M128	HERP-ΔUBL	pSG5-ZZ (M31)	ZZ	EcoRI/BamHI	M. Seeger
M240	HERP-hybrid	pSG5-ZZ (M31)	ZZ	EcoRI/BamHI	M. Seeger
M241	HHR23B	pSG5-ZZ (M31)	ZZ	EcoRI/BamHI	M. Seeger
M346	Ubiquilin-1	pSG5-ZZ (M31)	ZZ	BamHI	M. Seeger
M369	Ubiquilin-1-ΔUBL	pSG5-ZZ (M31)	ZZ	BamHI	M. Seeger
M243	RBX1	pSG5-ZZ (M31)	ZZ	BamHI	M. Seeger
M187	HERP	pSG5-ZZ (M31)	-	EcoRI/BamHI	M. Seeger
M188	HERP-ΔUBL	pSG5-ZZ (M31)	-	EcoRI/BamHI	M. Seeger
M229	HRD1	pSG5-ZZ (M31)	myc	EcoRI/BamHI	M. Seeger
M230	HRD1	pSG5-ZZ (M31)	myc	EcoRI/BamHI	M. Seeger
M301	CD3-delta	pCDNA3.1 hygro+	-	XhoI/XbaI	gift from M. Kikkert
M175	EGFPsiRNA-oligo	pSuper	-	EcoRI/HindIII	AS37/AS38
M207	HERPsiRNA-oligo	pSuper	-	EcoRI/HindIII	AS41/AS42
M307	HERPsiRNA-oligo	pSuper	-	EcoRI/HindIII	AS45/AS46
M309	HERPsiRNA-oligo	pSuper	-	EcoRI/HindIII	AS49/AS50

2.2 Protein biochemistry and immunology

2.2.1 Cell lysis

Unless otherwise noted mammalian cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, EDTA, 1% Nonidet P40, 0.5% Na deoxycholate, 0.1% SDS and Complete™ protease inhibitors (Roche)), incubated on ice for 10 min and centrifuged for 10 min at 4°C and 13,000x g. The supernatant was used for SDS-PAGE.

2.2.2 Determining protein concentrations

Protein concentrations in solution were determined photometrically by measuring the absorbance at 280 nm. An OD_{280} of one equals a concentration of 1 mg protein/mL solution. For a rough estimation of the protein concentration a spot test was performed. A standard BSA dilution series and the sample were pipetted onto Whatman 3MM paper and this was incubated in spot test staining solution (0.25% (w/v) Coomassie brillant blue G250, 10% (v/v) methanol and 7%

(v/v) acetic acid) for a few min. Destaining was performed in spot test destaining solution (10% methanol, 7% acetic acid).

2.2.3 TCA precipitation

For the precipitation of proteins from a cell lysate or after separating protein complexes on a glycerol gradient the trichloroacetic acid (TCA) precipitation method was chosen. A 50% TCA solution was added to the sample to yield a final concentration of 10% TCA. This mixture was kept on ice for 20 min, followed by centrifugation at 14,000x g and 4°C for 15 min. The supernatant was discarded and the pellet was washed with 250 µL ice cold acetone (-20°C). After another centrifugation step (5 min, 4°C, 14,000x g) the pellets were air dried and resuspended in 60 µL 1x SDS sample buffer.

2.2.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For the standard separation of proteins under denaturing conditions SDS-PAGE was performed using a discontinuous buffer system (Laemmli, 1970) with the vertical mini-Protean 3 electrophoresis system (Biorad). The concentration of acrylamide in the separating gels varied from 10-15%, depending on the resolution range desired. The stacking gel contained 5% acrylamide. The precise composition of the gels is given in table 6. The protein samples (2 µg pure protein or 100 µg cell lysate) were taken up in 5x sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.5% bromophenol blue, 0.5 M DTT), incubated at 95°C for 5 min, centrifuged and loaded onto the gel. Electrophoresis was carried out in 1x running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS) at 120 V. The size of the protein bands was estimated with a standard size marker, namely the prestained marker (NEB).

Table 6. Composition of SDS polyacrylamide gels. For two mini gels 1) acrylamide: bisacrylamid; 2) 1.5 M Tris-HCl pH 8.8, 0.4% SDS (w/v); 3) 0.5 M Tris-HCl pH 6.8, 0.4% SDS (w/v).

	Separating gel			**Stacking gel**
	10%	12%	15%	5%
acrylamide[1] 30:0.8 [mL]	6,7	8	10	3
H_2O [mL]	8,3	7	5	12
4x separating gel buffer[2] [mL]	5	5	5	-
4x stacking gel buffer[3] [mL]	-	-	-	5
TEMED [µL]	53	53	53	80
10% APS [µL]	53	53	53	80

2.2.5 Western blotting

After proteins were resolved by SDS-PAGE they were transferred onto a nitrocellulose membrane (Schleicher und Schuell) or a polyvinylidene fluorid (PVDF) membrane (Immobilon P, Millipore) in a semi dry blotting procedure. To do so, three Whatman 3 MM papers, the membrane, the gel and again three Whatman 3 MM paper sheets were piled up in a semi dry blotting apparatus (Peqlab). All components were soaked in Western transfer buffer (25 mM Tris, 200 mM glycine, 0.1% SDS und 20% methanol). The transfer was carried out at 400 mA for 1 h.

2.2.6 Protein staining procedures

Ponceau staining of proteins on nitrocellulose membranes

After the protein transfer onto nitrocellulose filters they were incubated in Ponceau staining solution (1% (w/v) Ponceau S in 5% (w/v) TCA-solution) for 3 min and thereafter rinsed in deionised water. Destaining was performed by shaking the membrane in PBST buffer.

Amido Black staining of proteins on PVDF membranes

PVDF membranes were incubated for 3 min in Amido Black staining solution (0.1% (w/v) Amido Black in 45% (v/v) methanol and 2% acetic acid) and rinsed in water until the protein bands were visible. Destaining was again performed by shaking the membrane in PBST buffer.

Coomassie Blue staining of proteins in polyacrylamide gels

Mini gels were immersed in Coomassie Blue staining solution (0.1% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, 10% (v/v) acetic acid, filtered before use) and shaken for at least 15 min. For the removal of unspecific staining the gels were incubated in destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid).

2.2.7 Immunodetection

Nitrocellulose- and PVDF membranes were incubated for at least 2 h in PBST buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 15 mM KH_2PO_4, 0.1% Tween 20) containing 5% milk powder followed by incubation with the primary antibody in PBST buffer for 2 h at RT. Thereafter, the blot membrane was washed four times for at least 10 min in PBST and subsequently incubated for 1 h with the secondary antibody coupled to horseradish peroxidase diluted in PBST. After the blot membrane was washed four times as before, detection was carried out as described in the BM Chemiluminescence Blotting Substrate kit (Roche). Visualisation was performed by exposure of the membrane to an Xomat-UV film (Kodak) for 1 s to 5 min.

Table 7. Primary antibodies for Western blot immunodetection.

Name	Origin	Dilution	Source
anti-β-Tubulin	mouse	1:10,000	Covance
anti-CD3-delta	rabbit	1:25,000	M. Seeger/Pineda Antikörper Service
anti-HERP	rabbit	1:100,000	M. Seeger/Pineda Antikörper Service
anti-HRD1 (C-term)	rabbit	1:1,000	Abgent
anti-Cdc48p (p97)	rabbit	1:20,000	kind gift from R. Hartmann-Petersen
anti-c-Myc	mouse	1:500	kind gift from A.A. Noegel
anti-VIMP	rabbit	1:1,000	kind gift from Y. Ye
anti-Derlin-1	rabbit	1:1,000	kind gift from Y. Ye
anti-Usp7 (BL851)	rabbit	1:10,000	Bethyl-Laboratories
anti-Penta-His	mouse	1:1,000	Qiagen
anti-β1 (20S proteasome)K43	rabbit	1:1,000	Peter-M. Kloetzel
anti-S1 (19S regulator)	mouse	1:1,000	Affinity Biomol
anti-S5a (19S regulator)	mouse	1:1,000	Affinity Biomol
anti-PA28α K39	rabbit	1:1,000	Peter-M. Kloetzel
anti-calnexin	mouse	1:8,000	BD Biosciences
anti-IgG (rabbit preserum)	rabbit	1:2,000	Pineda Antikörper Service

Table 8. Secondary antibodies for Western blot immunodetection.

Name	Origin	Dilution	Source
anti-rabbit-IgG-HRP	goat	1:5,000	Dianova
anti-mouse-HRP	sheep	1:5,000	Seramun

2.2.8 Preparation of microsomal membranes via differential centrifugation

For the crude preparation of microsomal membranes from whole cell extracts 2 x 10^9 Ramos cells (pellet volume of about 3 mL) were lysed in 3 mL microsome buffer (250 mM sucrose, 50 mM triethanolamine (TEA), 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 500 mM PMSF, 20 μM MG132) by physical shearing (20x) using a Dounce homogenisator on ice. The cell lysate was centrifuged (10 min, 1000x g, 4°C) to remove cell nuclei, unlysed cells and cell debris. The supernatant was again centrifuged twice as described. After a third centrifugation step the supernatant was centrifuged at 10,000x g for 10 min at 4°C. The resulting post-mitochondrial supernatant was centrifuged in an ultracentrifuge (TL-100, Beckman) at 100,000 rpm and 4°C for 1 h using a TLA55 rotor (Beckman). The microsomal pellet was solubilised in 500 μL microsome buffer shock frozen in liquid nitrogen and stored at -80°C.

2.2.9 Immunoprecipitation

Immunoprecipitation with ZZ-tagged fusion proteins

HeLa cells (4 x 10^6) were transfected with plasmids encoding ZZ and different ZZ fusions using the $CaCl_2$ method as described in section 2.3.5. Twenty-four hours after transfection the cells were lysed in Hepes buffer (33 mM Hepes pH 7.3, 150 mM potassium acetate, 10% (v/v) glycerol, 1% (w/v) DesoxyBigChap (Calbiochem) and Complete TM protease inhibitors (Roche)), gently agitated at 4°C for 30 min and centrifuged at 13,000x g for 10 min. The supernatant was transferred into a new tube and incubated with IgG Sepharose (Amersham) for 2 h to coprecipitate endogenous proteins. For co-precipitation studies with HRD1, cells coexpressing myc-tagged HRD1 and the different ZZ fusions were used. The beads were washed four times with lysis buffer lacking protease inhibitors but with 0.2% (w/v) DesoxyBigChap and 1 mg/mL BSA. Then, 20 units of TEV protease (Gibco) were applied to the sample to cleave the ZZ fusion between the ZZ tag and the protein and to release the complex into the supernatant. Supernatants were then separated via SDS-PAGE. Western blots of the immunoprecipitations prior to TEV cleavage were stained with rabbit preserum to show that comparable amounts of ZZ-fusion proteins were precipitated. Input lysates as well as precipitated proteins released from the IgG beads by TEV cleavage were incubated with antibodies as described in section 2.2.7.

Immunoprecipitation of intrinsic proteins using specific antibodies

Microsomal membranes from 2 x 10^8 Ramos cells (see section 2.2.8) or dog pancreas microsomal membranes were lysed in Hepes buffer as described above and supernatants were used for immunoprecipitation. The supernatants were incubated with specific antibodies as indicated in table 9 and gently agitated at 4°C for 4 h or over night. The immunocomplexes were precipitated with 30 µL packed Protein A-Protein G Sepharose (4:1, both from Amersham) for 2 h and subsequently washed four times for 5 min with 1 mL washing buffer (modified Hepes buffer with only 0.2% DBC and with 1 mg/mL BSA). The precipitated proteins were eluted by adding 50 µL 2x SDS sample buffer and detected via SDS-PAGE and Western blot analysis with specific antibodies (see table 7). In order to reduce the signal of IgG heavy and light chains from the immunoprecipitation during immunodetection, Protein A-HRP (Biorad) was applied in a dilution of 1:2,000 instead of the secondary antibody. Immunoprecipitation with solubilised dog pancreas microsomes was performed by Yihong Ye, Harvard Medical School, Boston.

Table 9. Antibodies for immunoprecipitation.

Name	Origin	Dilution	Source
anti-HERP affinity purified	rabbit	1:10	Pineda
anti-HRD1	rabbit	1:10	Abgent
anti-VCP (p97)	mouse	1:200	Affinity BioReagents
anti-Vimp	rabbit	1:200	kind gift from Y. Ye
anti-Derlin-1	rabbit	1:200	kind gift from Y. Ye
anti-α6 (proteasome)	rabbit	1:200	Peter-M. Kloetzel

2.2.10 Glycerol gradient centrifugation

HeLa cells (10^7) transfected with plasmids encoding HRD1-myc or HRD1-(C329A)-myc 24 h prior to cell lysis or untransfected Ramos cells (2 x 10^7) were lysed in a modified Hepes buffer (see section 2.2.9) containing only 5% gycerol. The lysate supernatants were separated via centrifugation on a glycerol gradient ranging from 10-50% based on the lysis buffer. Centrifugation was performed using an ultracentrifuge (Ultra Pro80, Sorvall) for 16 h at 40,000 rpm and 4°C with an SW40 rotor (Beckman Coulter). The gradient was divided into fractions of 900 µL, which were TCA precipitated and solubilised in 60 µL 1x SDS buffer. Samples were characterised via SDS-PAGE and immunodetection.

2.2.11 *In vitro* binding assays

Protein expression

For the expression of GST-tagged recombinant fusion proteins (table 4) in *E. coli* the bacterial strain BL21 De3 was used. Cells were transformed with the respective expression vectors and 1/10 of the transformed cells was streaked out on LB_{amp} agar plates and incubated over night at 37°C. The next day, 10 mL liquid LB_{amp} medium were inoculated with a single colony and shaken over night at 37°C. Then 200 mL LB_{amp} were inoculated with the 10 mL culture and grown to an OD_{600} of 0.5. Protein expression was induced by the addition of 0.4 mM IPTG. The cultures were then incubated at RT for 4 h and aliquots were taken at different time points to recapitulate the protein expression via SDS-PAGE and Coomassie staining.

Cell lysis and binding of GST fusion proteins to glutathione Sepharose

The *E. coli* cells containing the protein of interest were sedimented by centrifugation (5,000x g, 4°C, 30 min) and lysed in 5 mL GST lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.3, 100 mM NaCl, 10% glycerol, 1 mM PMSF, Complete™ protease inhibitors, 1 mg/mL lysozyme (Fluka)), sonicated four times for 30 s and gently agitated for 1 h at 4°C. Lysates were centrifuged

for 30 min at 14,000x g and supernatants were transferred into a fresh Falcon tube. Precipitation of GST fusion proteins was performed by incubation with 300 µL glutathion Sepharose (Amersham, 1:1 in GST-binding buffer: 1% Triton X-100, 50 mM Tris-HCl pH 7.3, 100 mM NaCl, 10% glycerol) for at least 2 h on a rotor. Glutathione Sepharose beads were washed four times with GST binding buffer containing 1 mM PMSF, solubilised in 1 mL GST lysis buffer without lysozyme and stored on ice.

Preparation of *E. coli* cell lysates containing His-tagged fusion proteins

Expression of His-tagged proteins was performed with pQE30 (Qiagen) vectors and the *E. coli* strain M15 as described above. Cells were lysed in 5 mL Hepes buffer (1% DBC, 33 mM Hepes pH 7.3, 150 mM potassium acetate, 4 mM magnesium acetate, 10% (w/v) glycerol, Complete ™ protease inhibitors and 1 mM PMSF), sonicated and incubated for 1 h at 4°C with gentle agitation. Lysates were centrifuged for 30 min at 14,000x g and the supernatants were used for *in vitro* binding assays. For the incubation of immobilised GST fusion proteins with cell lysates from human cells, 5 x 10^6 HeLa cells or 10^7 Ramos cells were lysed in 1 mL Hepes buffer, incubated for 30 min at 4°C and centrifuged for 10 min at 14,000x g and 4°C. The supernatants were then utilised for the pull-down assays.

***In vitro* pull-down assays with human or *E. coli* cell lysates**

For *in vitro* pull-downs 20 µL of GST fusion proteins bound to glutathione Sepharose were incubated with 500 µL *E. coli* lysates or 1 mL human cell lysates for 2 h or over night with gentle agitation. After extensive washing with Hepes buffer containing 0.2% DBC and 1 mg/mL BSA the beads were taken up in 60 µL of 2x SDS sample buffer and the protein complexes bound to the beads were analysed via SDS-PAGE and immunodetection.

2.2.12 Affinity purification of antibodies

Purification of antibodies was performed with a HiTrap NHS column from Amersham. GST-HERP-ΔC was first expressed in BL21 *E. coli* cells, bound to glutathione Sepharose and then eluted from the beads with 150 µL elution buffer (0.2 M $NaHCO_3$ pH 8.3, 0.5 M NaCl, 10 mM GSH reduced and 5 mM β-mercaptoethanol). The Sepharose was sedimented, the supernatant transferred to a new tube and 150 µL elution buffer was added once more. After repeating this step a third time the three supernatants were pooled and the efficiency of the elution was determined via SDS-PAGE followed by Coomassie staining. The protein yield was determined by spot test. Prior to coupling the protein to the column, isopropanol was washed off the column

with 10 mL 1 mM ice cold HCl. Then, 0.25 mg eluted GST-HERP-ΔC was diluted 1:1 in coupling buffer (0.2 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) and injected onto the column. The flowthrough was collected and injected again. After repeating this step four times the column was washed alternately with 6 mL of blocking buffer (0.2 M $NaHCO_3$ pH 8.0, 0.5 M NaCl) and 6 mL wash buffer (0.5 M NaCl pH 4.0, 0.5 M ethanolamine). After every second washing step with blocking solution the column was incubated at RT for 15 min. After the column was washed the fourth time with blocking solution 5 mL HERP serum were injected onto the column. The flowthrough was collected and again loaded onto the column for four times. Then the column was washed with 10 mL equilibration buffer (0.01 M Na_2HPO_4, 0.14 M NaCl). The elution was performed with 5 mL elution buffer (0.1 M glycine pH 3.0, 0.5 M NaCl). Fractions of 500 µL were collected and neutralised with 10% 1 M Tris-HCl pH 8.0. To determine which fraction contained the antibody a Western Blot was performed with HeLa lysates and incubated with an aliquot of each fraction in a 1:1,000 dilution. Positive fractions were stored in aliquots of 50 µL at -20°C.

2.3 Tissue culture

2.3.1 Cell lines and media

In this study HeLa human cervix carcinoma cells and Ramos human B cell lymphoma cells were used. HeLa cells were cultivated in Iscove, Ramos cells in RPMI medium (both Biochrom) with 10% heat inactivated fetal bovine serum (Biochrom), 2 mM L-glutamine (Biochrom), and 100 U/mL penicillin, 100 µg/mL streptomycin (Seromed). Cells were grown at 37°C and 5% CO_2.

2.3.2 Culture of adherent cells and cells in suspension

Cells were grown in sterile culture dishes or flasks (Greiner) and passaged every two to three days depending on the generation time. For the passage of adherent cells the culture medium was removed, the cells were washed with PBS and incubated with a 1:4 dilution of a trypsin/EDTA solution (GibcoBRL) in PBS for 2-5 min. After detachment of the cells the reaction was stopped by addition of culture medium containing serum. The cells were plated in an appropriate density.

10x PBS: 1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4

2.3.3 Permanent culture and thawing of mammalian cells

In order to prepare a permanent culture of human cell lines these were detached from the culture dish with trypsin/EDTA and sedimented (1,000 rpm, 5 min). Two x 10^6 cells were resolved in 1 mL of medium supplemented with 20% FCS and 10% 2-methylbenzyl chloride (DMSO, Sigma), immediately transferred into a cryo tube (Nunc) and placed in a freezing container (Cryo 1°C, Nalgene) filled with pre-cooled isopropanol. The container was kept at -80°C over night to ensure a 1°C/min cooling rate. The next day the tubes were transferred into a liquid nitrogen (-196°C) tank for long-term storage.

Thawing of cells was performed in a 37°C water bath. The suspension was transferred from the cryo tube into 10 mL culture medium and sedimented at 4°C and 1,000x g for 5 min. The culture medium was carefully removed and the cells were resuspended in medium and resedimented. They were plated in 10 mL culture medium on a 10 cm culture dish.

2.3.4 Counting of cells

Ten µL of a single cell suspension were inspected with a Neubauer counting chamber under a microscope. Cells in four selected large squares were counted. The volume of one large square is 0.1 µL. The mean value of four large squares was multiplied with 10^4, resulting in the number of cells per 1 mL cell suspension.

2.3.5 Transfection of HeLa cells

HeLa cells were transfected either by lipofection, by the calcium phosphate method or by electroporation. For transfection with siRNAs the HighPerFect reagent (Qiagen) was used.

Transfection with Lipofectamin 2000

HeLa cells (10^5) were plated in a 24 well plate in 500 µL medium without antibiotics and incubated over night. For each well 0.8 µg DNA and 0.8 µL Lipofectamin 2000 (Invitrogen) were diluted in 50 µL OptiMEM (Invitrogen), mixed, incubated at RT for 20 min and then transferred to the well containing the cells. The cells were then incubated at 37°C for 24 h after plasmid transfection or for 48 h after siRNA transfection.

Calcium phosphate transfection

HeLa cells were plated at 0.75 x 10^6 cells in a 10 cm dish in growth medium and incubated over night at 37°C. On the next day 2x HBS buffer (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM Hepes pH 7.05) was pipetted into a 5 mL polypropylene Falcon tube

(Greiner, # 2063). Then, 62 µL 2 M $CaCl_2$ and 10 µg DNA were diluted in 440 µL sterile water, which was then very slowly dripped into the tube containing the 2x HBS buffer while vortexing the tube on the slowest setting. The precipitate was incubated at RT for 30 min, mixed and dripped onto the plate containing the cells. The plate was gently shaken to distribute the DNA and incubated at 37°C. After 16 h the medium was exchanged and after 24 h the cells were harvested.

Electroporation of HeLa cells

HeLa cells (5 x 10^6) were trypsinised and collected by centrifugation. The cells were resuspended in 300 µL medium and transferred into an electroporation cuvette with 4 mm electrode gap (PeqLab). Twenty µg DNA were added to the cells and these were kept on ice for 10 min. The electroporation was performed at 975 µF and 210 V in an Easyject Optima electroporator (Equibio). After 5-10 min incubation at RT the cells were plated on a 10 cm culture dish and incubated at 37°C. After attachment of the cells to the dish the medium was exchanged to remove dead cells. In most cases cells were analysed 40 h after transfection.

Transfection of siRNAs with the HiPerFect reagent

HeLa cells (6 x 10^4) were plated in a 24 well plate in 500 µL medium and incubated over night at 37°C. The siRNAs (150 ng) were mixed with 4.5 µL HiPerFect reagent (Qiagen) in 100 µL medium without serum and incubated for 10 min at RT. The solution was mixed and dripped onto the plate containing the cells and incubated for 24 h at 37°C. For co-transfection of siRNAs with plasmids 100 ng DNA and 150 ng siRNA were diluted in 25 µL HBS buffer (20 mM Hepes, 150 mM NaCl, pH 7.4). In parallel, 4.5 µL HiPerFect reagent were mixed with 21.5 µL HBS buffer, added to the nucleic acids, gently vortexted and incubated at RT for 10 min. The solution was applied to the cells as described and these were incubated for 24 h at 37°C.

Table 10. Sequences for siRNA mdiated downregulation of USP7 (Qiagen).

Name	Target sequence
Hs_USP7_1_HP siRNA	CGGGCCGACACCAGTACATAA
Hs_USP7_2_HP siRNA	ATGGAGTTGCGTGGGATTCAA
Hs_USP7_3_HP siRNA	CCCAAATTATTCCGCGGCAAA
Control (non-silencing) siRNA	AATTCTCCGAACGTGTCACGT

2.3.6 Generation of stable HeLa cell lines via puromycin selection

For the generation of cell lines stably expressing the HERP protein and a HERP variant lacking its UBL domain HeLa cells were co-transfected with 10 μg of the corresponding expression vectors and 2 μg of a vector coding for a puromycin resistance gene using the calcium phosphate method. Two days after transfection the cells were detached and replated in a 96 well plate in different dilutions from 5 x 10^4 to 10^2 cells per well. The culture medium contained 2 μg/mL puromycin (Fluka) to select for clones carrying a stable insertion of plasmid DNA in the genome. Only these cells formed colonies within two weeks. Colonies derived from a single cell were expanded. A cryostock was generated of each clone, as described in section 2.3.3. To screen for clones that expressed the transgene, samples were analysed by immunoblotting with a HERP-specific antibody.

2.3.7 Apoptosis assays

Determination of the caspase-3/7 activity

The caspase-3/7 activity was determined with the Apo-ONE™ Homogeneous caspase-3/7 Assay (Promega). To do so, 10^4 HeLa cells were plated in each well of a 96 well plate with black sides but transparent base and incubated over night. The next day the cells were transfected with the siRNA plasmids using Lipofectamine 2000. Another 24 h later ER stress was induced using 10 μg/mL tunicamycin (Sigma). The caspase-3/7 activity was determined in 50 μL of culture medium. Z-DEVD-R110 substrate was mixed 1:100 with the buffer and 50 μL of this solution were pipetted onto the cells. After 30 min to 3 h incubation in the dark with gentle agitation at RT the caspase-3/7 activity was determined with the Fluorimeter Flurostar Reader with Easy Software (SLT/Tecan) at 485 nm (excitation) and 538 nm (emmission).

Caspase-3/7 cleavage assay

Cleavage of the HERP protein by a caspase-3/7 activity upon prolonged ER stress was determined using the caspase-3/7 inhibitor Z-DEVD-FMK (BD Biosciences). 1.0 mg of the inhibitor was dissolved in 150 μL DMSO resulting in a 10 mM stock solution. HeLa cells were treated with 10 μg/mL tunicamycin to induce ER stress. At the same time the caspase-3/7 inhibitor was added to a final concentration of 10 μM. Equivalent volumes of the solvent DMSO were added to the control samples. Cells were lysed with RIPA buffer at various time points and HERP cleavage was analysed by Western blotting.

2.3.8 Cycloheximide chase

For the determination of the half life of different proteins 10^5 HeLa cells synthesising the protein of interest were plated on a 24 well dish. On the next day cycloheximide was added to a final concentration of 10 μg/mL. In some experiments 10 μg/mL tunicamycin were added to the cells 4 h prior to cycloheximide addition to induce ER stress. Then, cycloheximide was added and the cells were harvested at various time points and immediately lysed with RIPA buffer. Where indicated the proteasomal inhibitor MG132 (Calbiochem) was added to a final concentration of 10 μM. In these cases the other samples contained the equivalent volume of the solvent DMSO. Samples were analysed by Western blotting.

2.3.9 Immunofluorescence of cultured cells

HeLa cells grown on cover slips were transfected with constructs encoding different EGFP-tagged MUBL constructs using Lipofectamin 2000 and incubated 12 to 24 hours at 37°C. The cells were washed with PBS and 200 μL ER tracker™ Red dye (Molecular Probes) was added in a dilution of 1:1000 in PBS. ER tracker™ Red is a drug conjugate of glibenclamide BODIPY®, with excitation/emision maxima of 587/615 nm. The drug glibenclamide binds to the sulphonylurea receptors of ATP-sensitive K^+ channels, which are prominent on the ER membrane. The cells were incubated at 37°C for 10 min and subsequently fixed with 3.7% paraformaldehyde in PBS. For fixation cells were incubated at 37°C for 2 min and washed twice more for 5 min with PBS. Mounting medium was applied and the coverslips were inverted and placed on glass slides. Immunofluorescence was documented with a Leitz DMRD microscope from Leica.

Mounting medium: 2.4 g Mowiol 40-88, 6 g glycerol and 6 mL H_2O stirred for several hours at RT; 12 mL 0.2 M Tris-HCl pH 8.5 added and incubated at 50°C for 10 min; centrifugated (5,000x g, 15 min) to pellet non-diluted Mowiol

2.4 Bioinformatics and database analysis

Different bioinformatics tools and databases were used during this study. Tasks included the analysis of sequencing data after cloning of different genes into expression vectors, designing primers for semi-quantitive RT-PCRs, homology searches, the prediction of transmembrane helices and the subcellular localisation of proteins as well as extensive literature searches were performed. Tools and databases for these studies and their world wide web adresses are listed below.

National Center for Biotechnology Information, Bethesda, MD, USA
PubMed, UniGene, Nucleotide, Blast Search, Blast 2 Sequences (http://www.ncbi.nlm.nih.gov/)

Swiss Institute for Bioinformatics, Switzerland
Swiss-Prot - Protein Knowledgebase (http://www.expasy.org/sprot/)

European Bioinformatics Institute, Hinxton, UK and The Wellcome Trust Sanger Institute, Hinxton, UK
Ensembl (http://www.ensembl.org/)

The Wellcome Trust Sanger Institute, Hinxton, UK
Protein families database of alignments and HMMs (Pfam)
(http://www.sanger.ac.uk/Software/Pfam/)

The design of RT-PCR primers was carried out with Primer 3
(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi).

Sequence alignments and phylogenetic tree analyses were performed with:
Bioedit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html)
ClustalW (http://www.expasy.org/sprot/)
TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html)

For densitometric analyses of Western blots ImageJ (http://rsb.info.nih.gov/ij/) was utilised.

For prediction of suitable siRNA sequences to be cloned into pSuper vectors the target sequence finder (http://www.ambion.com/techlib/misc/siRNA_finder.html) was used.
Sequences were checked for unique target sequences with the program BLAST (http://www.ch.embnet.org/software/bBLAST.html).

For screening proteins containing both UBL domains as well as transmembrane helices the program SMART (http://smart.embl-heidelberg.de) was used.

3 RESULTS

3.1 HERP and related human UDPs - initial characterisation of a novel protein family

3.1.1 HERP and other human UDPs containing transmembrane helices

HERP is a human UDP, which contains transmembrane helices, that anchor the protein in the ER membrane. Interestingly, HERP was shown to be upregulated upon the accumulation of unfolded proteins in the ER by the unfolded protein response (UPR). However, its role within the UPR was unknown.

Some human UDPs such as ubiquilin-1 or HHR23B have paralogues with overlapping functions in the cell. These paralogues can therefore take over each others function. Paralogues of the HERP proteins have not been identified so far. Thus, utilising the SMART program a database search was performed to screen for human proteins containing both a UBL domain and potential transmembrane regions. In this screen four proteins, including HERP, were identified. Among these proteins HERP was the only one that was known from the literature. Thus, apart from HERP the human genome encodes at least three other proteins containing a UBL domain and transmembrane regions. Accordingly, these proteins were designated as MUBLs, for transmembrane-associated protein containing a ubiquitin-like domain. The domain architecture of these proteins is depicted in figure 7.

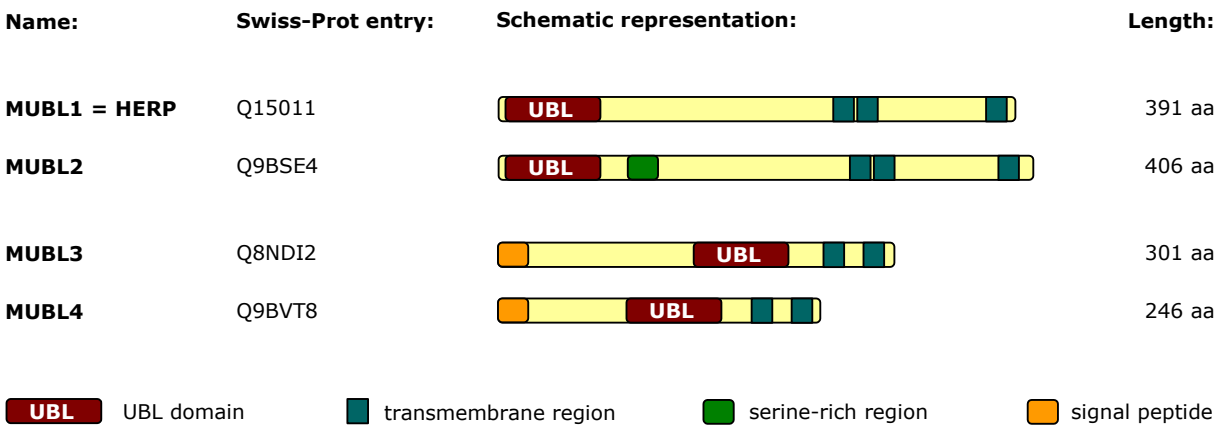

Figure 7. Domain architecture of the four human MUBL proteins. Schematic representation of the domain architecture of HERP and the three other MUBLs. The different domains are colour-coded according to the key below.

The four human MUBLs fall into two groups according to their structural features (Fig. 7). These groups are not related to each other. However, the proteins belonging to the same group display considerable similarity. HERP and MUBL2 both consist of an N-terminal UBL domain as well as potential transmembrane helices at the C-terminus. In addition, MUBL2 contains a serine-rich region downstream of its UBL domain, which is not present in HERP. Their amino acid sequences are 40% identical. MUBL3 and MUBL4 share 37% similarity in their protein sequences. Both these proteins contain a signal sequence at the N-terminus and potential transmembrane helices at the C-terminus. Their UBL domains, in contrast to those in most other UDPs, are located in the middle of the primary structures.

Database searches for orthologues of HERP and the three related human MUBLs identified orthologues in *Mus musculus* and *Rattus norvegicus*. The amino acid sequences of these orthologues are at least 85% identical to their human counterparts. However, orthologues of the MUBL proteins were not found in other organisms. Therefore, these four UDPs appear to exist exclusively in mammals.

3.1.2 Sequence analyses of the UBL domains of the MUBLs and other human UDPs

An alignment of the UBL domains of HERP, the three other MUBLs and various additional human UDPs (Fig. 8A) demonstrated that the different UBL domains share only little sequence similarity. This rather weak sequence similarity is also reflected by the phylogenetic tree in figure 8B. The UBL domains of paralogue proteins are more similar to each other. The four ubiquilins, for instance, are orthologues of the yeast protein Dsk2p and their UBL domains share strong sequence identity (92% identity for ubiquilin-1 and -2 on amino acid level). Furthermore, the UBL domains of HHR23A and HHR23B share 79% sequence identity, as they are both orthologues of the yeast Rad23p.

A rather strong sequence similarity also exists between the UBL domains of HERP and MUBL2, which share 53% sequence identity while they differ in sequence from those of MUBL3, MUBL4 and other UDPs. The UBL domains of MUBL3 and MUBL4 are also similar with 57% identity. These findings support the similarities found for the full length protein sequences of the MUBLs. Additionally, this suggests that HERP and MUBL2 are paralogues, which might take over each others functions in the cell. The same is true for MUBL3 and MUBL4, which are structurally related but differ in sequence and structure from HERP and MUBL2.

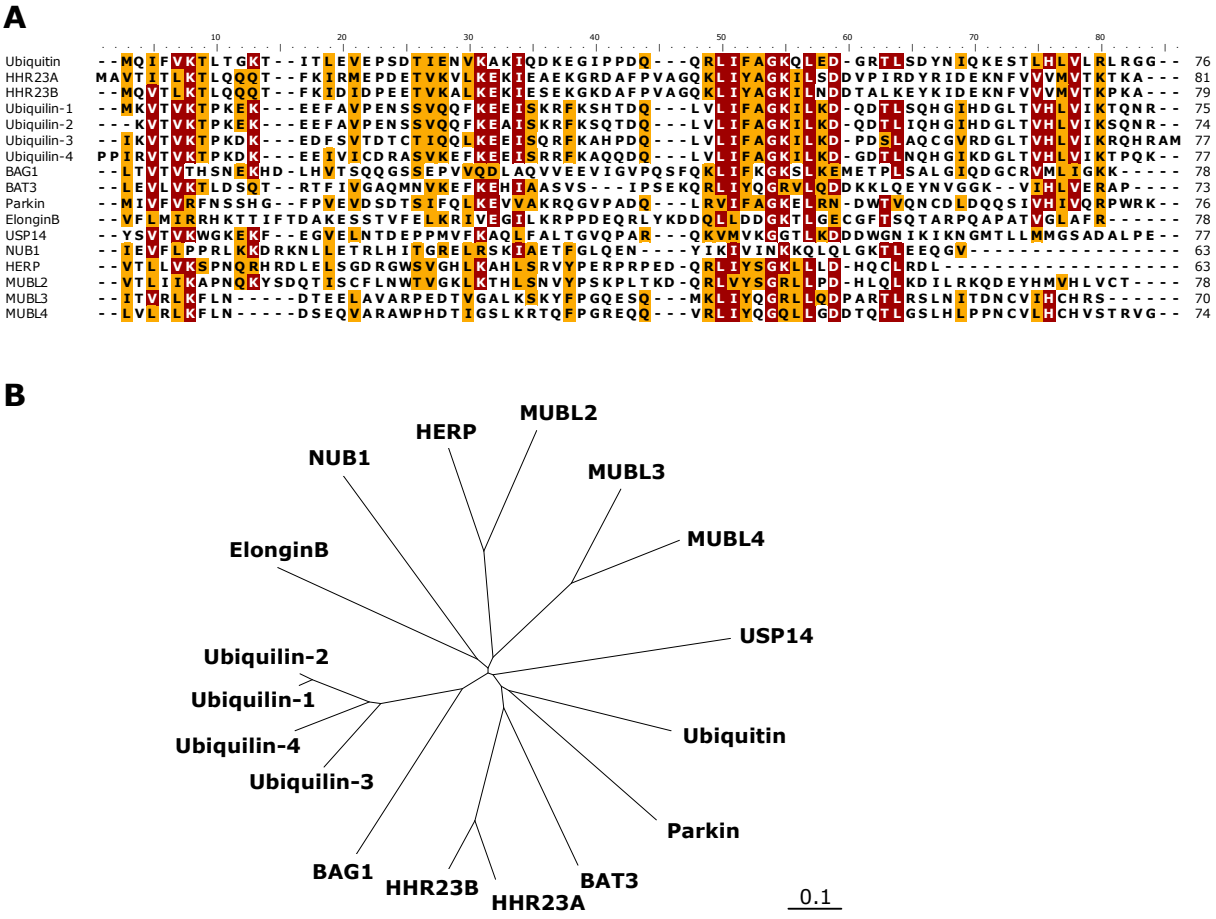

Figure 8. Primary structures of selected human UBL domains. (A) Alignment of amino acid sequences of the UBL domains of HERP, MUBL2, MUBL3 and MUBL4 as well as selected UDPs. Identical amino acids are highlighted in dark red, similar amino acids in orange. **(B)** Phylogenetic tree of the UBL domains. The scale bar is a measure for amino acid substitutions per residue.

3.1.3 Subcellular localisation of MUBL3 and MUBL4

HERP was shown to be an integral membrane protein, which localises to the ER with both the N- and the C-terminus facing the cytosol (Kokame et al., 2000; van Laar et al., 2000). MUBL2 was predicted to possess a similar membrane topology as HERP with two hydrophobic regions at the C-terminus of the protein (Fig. 7). MUBL3 and MUBL4, in contrast, have two C-terminal hydrophobic patches and a potential signal sequence at the N-terminus (Fig. 7). This potential signal sequence and the transmembrane regions suggest that these proteins might also associate with the ER. Thus, colocalisation studies with EGFP-tagged fusion proteins of all four MUBLs with an ER marker were performed. Unfortunately, EGFP-tagged HERP and MUBL2 had a strong tendency to form aggregates when expressed in HeLa cells. This was most likely due to

the EGFP tag, which is known to sometimes impede with the proper folding of the fused proteins. Thus, the subcellular localisation of HERP and MUBL2 cound not be studied with this approach. However, the EGFP tag did not seem to prevent the folding of MUBL3 and MUBL4 fusion proteins. They were both localised to the ER, as can be seen by the strong overlap of EGFP with the ER marker (Fig. 9). Thus, although further experiments are required to unambiguously clarify the subcellular localisation of these proteins, this data suggests that MUBL3 and MUBL4 are localised to the ER.

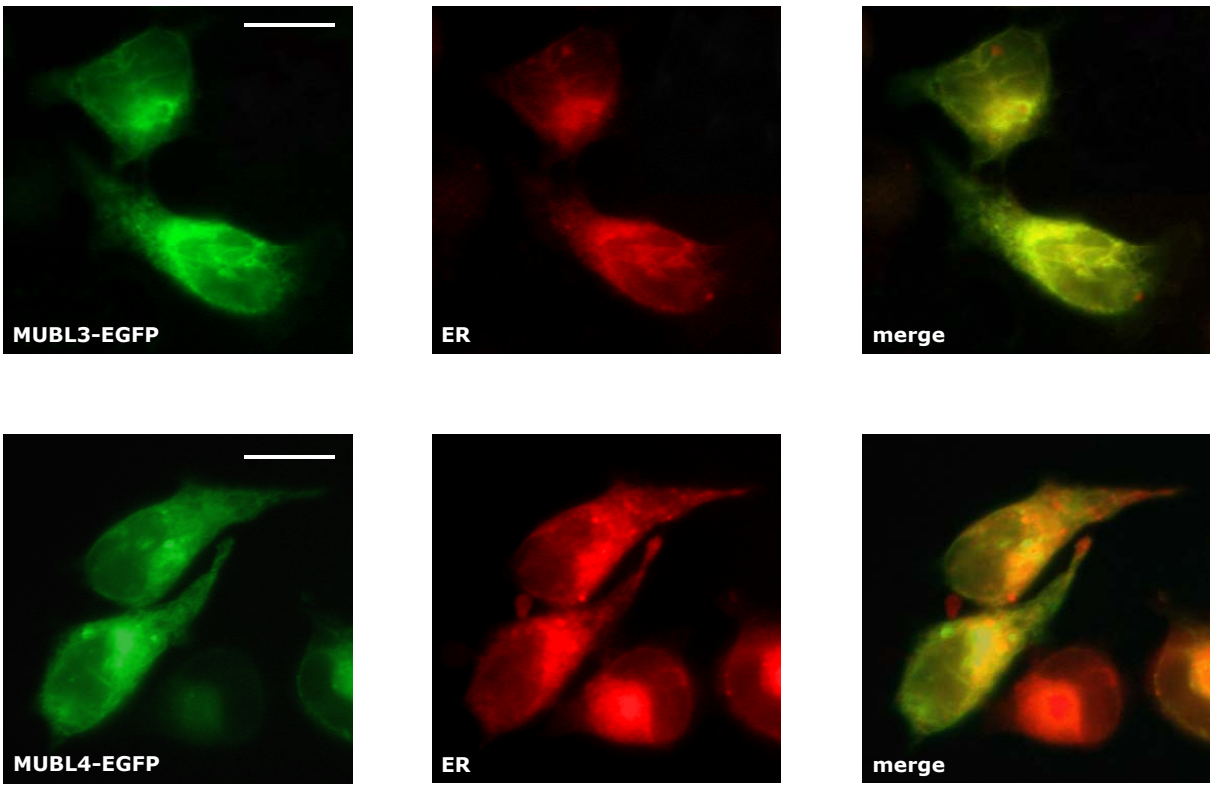

Figure 9. Subcellular localisation of EGFP-tagged MUBL3 and MUBL4. HeLa cells were transfected with plasmids coding for C-terminally EGFP-tagged MUBL3 (upper panels) or equally tagged MUBL4 (lower panels), and stained with ER tracker™. The left panels show the EGFP fluorescence of the MUBL3-EGFP and MUBL4-EGFP fusions, the middle panels the ER staining with ER tracker™ and the left panels the merged images (scale bars equal 20 μm).

3.1.4 Tissue distribution of *MUBL2* mRNA

The strong similarity of HERP and MUBL2 suggested that these proteins might have related functions and might be able to compensate for each other in the cell. Thus, to get an idea of the role of MUBL2, the distribution of *MUBL2* mRNA in different human organs was examined by

Northern blot analysis (Fig. 10). *MUBL2* transcripts were most abundant in brain and spleen, but were also detected in pancreas, placenta, small intestine and thymus. *MUBL2* mRNA was not detected in heart, liver, lung and kidney, although the total RNA representing kidney was partially degraded. In contrast to the expression of *MUBL2*, *HERP* mRNA was ubiquitously expressed in the eight organs tested, namely heart, brain, placenta, lung, liver, sceletal muscle, kidney and pancreas (Kokame et al., 2000). *HERP* encoding transcripts were most abundant in pancreas, and low in brain, lung and kidney. Thus, the expression patterns of *HERP* and *MUBL2* vary from each other, which suggests that HERP and MUBL2 have different, tissue specific functions. While *HERP* transcripts are most abundant in organs with high secretory activity such as pancreas, enhanced *MUBL2* mRNA levels can be found in tissues important for the immune response of the organism. Therefore, it is feasible that MUBL2 has a role in the immune response.

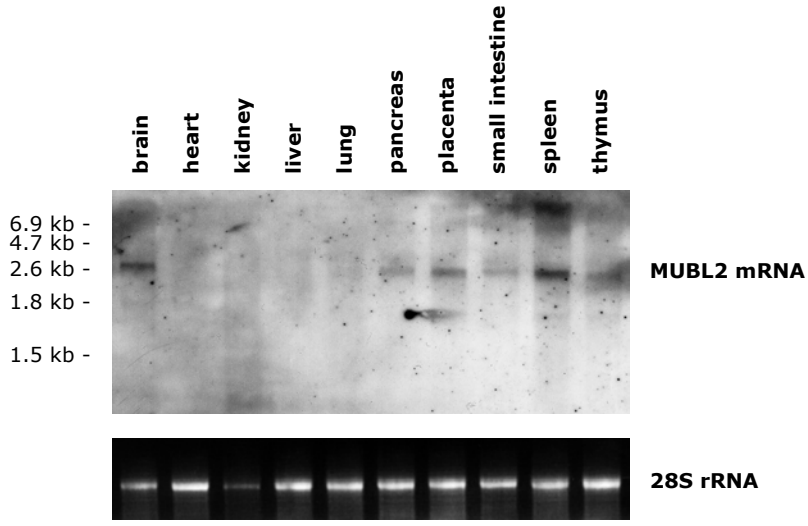

Figure 10. Distribution of *MUBL2* mRNA in human organs. A Northern blot was performed with human total RNA (2 μg total RNA/lane) and hybridised with a DIG-labelled *MUBL2* RNA probe (upper panel). Positions of standard RNA markers are shown on the left with their sizes. As a loading control, the 28S rRNA from the gel is shown in the lower panel.

3.1.5 *MUBL2*, *MUBL3* and *MUBL4* are constitutively expressed during the UPR and interferon response

A number of human genes whose products are involved in protein folding in the ER or in ERAD are induced by the UPR. Although the function of HERP was unknown so far, it was shown to be upregulated by this pathway (Kokame et al., 2000; van Laar et al., 2000). Thus, this might also be true for MUBL2, -3 or -4. To elucidate this, HeLa cells were treated with the

reducing agent β-mercaptoethanol to induce the UPR and mRNA levels of *HERP*, *MUBL2*, *MUBL3* and *MUBL4* were measured by reverse transcription polymerase chain reaction (RT-PCR). As known from the literature a marked increase was observed in the *HERP* transcript levels in response to ER stress in this experiment (Fig. 11). However, the mRNA levels of *MUBL2*, *MUBL3* and *MUBL4* did not change during the UPR in comparison to untreated cells. Thus, the genes encoding the three novel MUBLs were constitutively expressed during the UPR. Since *MUBL2* transcripts were shown to be present in organs important for immune response (Fig. 10), its expression might be induced during the interferon response. To test this possibility, HeLa cells were treated with interferon-γ and the mRNA levels of the four MUBL proteins were measured as described above (Fig. 11). None of the tested genes were induced after treatment with interferon-γ. Therefore, *HERP*, *MUBL2*, *MUBL3* and *MUBL4* expression was unchanged during the interferon response in HeLa cells.

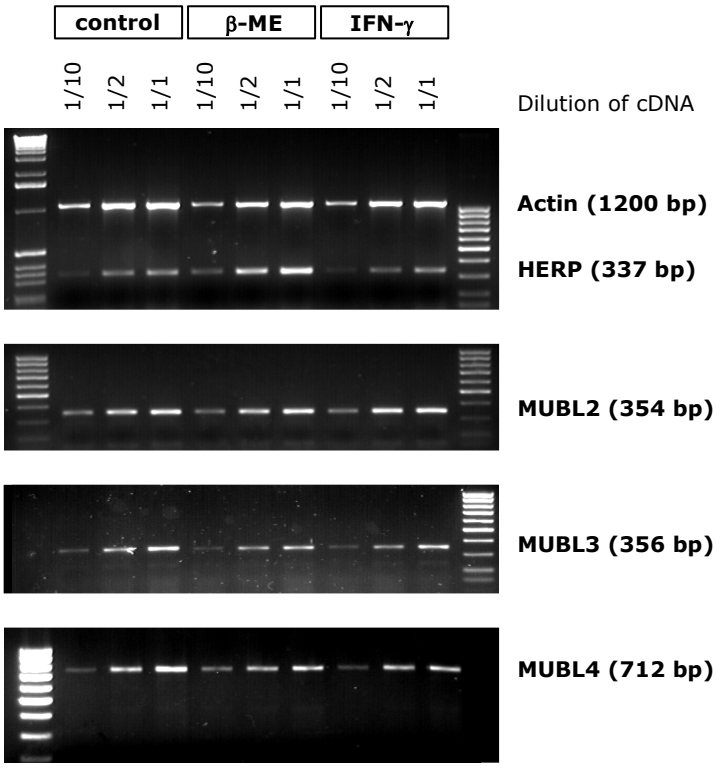

Figure 11. Expression levels of HERP and the three other MUBLs upon ER stress and stimulation with interferon-γ. Untreated HeLa cells or HeLa cells that were treated with 10 mM β-mercaptoethanol (β-ME) for 4 hours or with 200 U/mL interferon-γ (IFN-γ) for 24 h were harvested and analysed for the presence of specific mRNAs for HERP, MUBL2, MUBL3, MUBL4 via RT-PCR. Primers for Actin were used as an intrinsic control in the HERP samples. As an additional control measure, three different dilutions of cDNA were used. Positions of standard DNA markers are shown on the left and on the right.

3.2 The role of the ubiquitin domain protein HERP within the UPR

3.2.1 Generation and characterisation of HERP-specific siRNAs

The initial experiments demonstrated that HERP is the only one of the four MUBL proteins whose transcript levels are enhanced by the UPR. To elucidate whether HERP has a role within ERAD, protein synthesis of HERP was reduced with small interfering RNAs (siRNAs). siRNAs are 21-nucleotide RNA duplexes that are homologous in sequence to an endogenous gene and specifically suppress the expression of this gene (Elbashir et al., 2001). Three different 21-nucleotide sequence fragments of the *HERP* gene were chosen according to siRNA sequence prediction algorithms and cloned into the pSuper vector (table 11). A sequence derived from the *EGFP* gene was chosen as control.

Table 11. Sequences tested for siRNA mediated downregulation of HERP synthesis

#	Sequence starting point [bp]	Sequence	Efficiency
1	300	ATCAACGCCAAGGTGGCTG	+
2	183	ACATCTCAAGGCCTGAAGC	++
3	380	ATGATGGTCCTCCTCCTGA	+

In order to test the efficiency of HERP downregulation by these three sequences, HeLa cells were transfected with the HERP pSuper constructs and the EGFP control plasmid. At 48 h after transfection lysates of the cells were subjected to SDS-PAGE and Western blot analysis with an anti-HERP antibody (Fig. 12A). Cells were left untreated or were treated with tunicamycin 4 h prior to lysis. Tunicamycin inhibits the N-glycosylation of ER proteins leading to an induction of the UPR and therefore to the synthesis of the HERP protein. As demonstrated in figure 12, transfection of all HERP pSuper constructs resulted in a downregulation of HERP synthesis. After transfection with the HERP pSuper construct #1, HERP protein was reduced to 70% compared to control. Addition of tunicamycin led to an increased efficiency with a reduction of the HERP protein content to 30% of control value. Transfection of the HERP pSuper construct #2 resulted in a downregulation to 40% or 50% of the protein in the absence or presence of tunicamycin, respectively. With the HERP pSuper construct #3 the silencing effect was less drastic. Since it was important for some experiments to obtain an efficient downregulation without HERP induction by tunicamycin the HERP pSuper construct #2 was chosen for all following experiments.

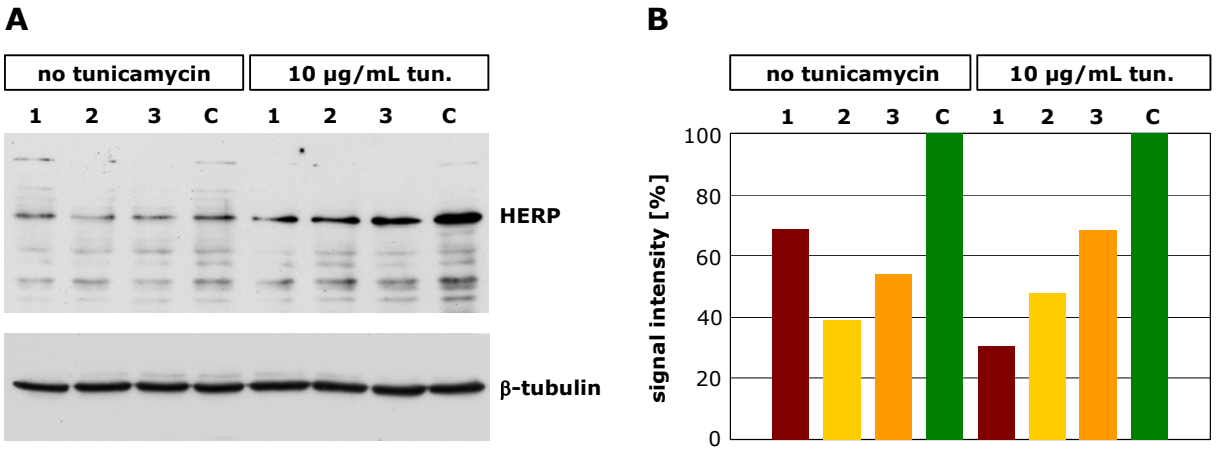

Figure 12. siRNA-mediated downregulation of HERP synthesis by HERP-specific sequences. (A) HeLa cells were transfected with pSuper constructs specific for HERP. At 48 h after transfection the cells were harvested and lysed with RIPA buffer. Where indicated cells were treated with tunicamycin 4 h prior to cell lysis to induce ER stress. Twenty µg of lysate were subjected to SDS-PAGE and Western blot analysis with antibodies specific for HERP or β-tubulin. Three different sequences were tested (see table 10). As a control (c) a sequence from enhanced green fluorescent protein (EGFP) was used. **(B)** Densitometric analysis of the Western blot from (A). The signal intensity of the control was defined as 100% to compare the silencing efficiency of the different HERP sequences. tun. = tunicamycin.

3.2.2 HERP rescues the cell from early activation of ER stress-induced caspase-3/7 activity

Prolonged exposure of cells to ER stress leads to caspase-3-mediated apoptosis (Rutkowski and Kaufman, 2004). The induction of this ER stress-induced apoptosis can be inhibited by the overexpression of certain ER chaperones or ERAD components. For instance, overexpression of the E3 ligase HRD1 was shown to protect cells from UPR-induced apoptosis since it is required for ubiquitylation of ERAD substrates and thus involved in the clearance of accumulated proteins from the ER. Concomitantly, an siRNA-mediated downregulation of HRD1 expression resulted in early onset of apoptosis (Amano et al., 2003; Kaneko et al., 2002). Being upregulated by the UPR HERP might also contribute to the survival of the cell during ER stress. To address this, a caspase-3/7 activity assay was performed with HeLa cells expressing either HERP or EGFP siRNAs to monitor the onset of ER stress-induced apoptosis. ER stress was induced by the addition of tunicamycin and since prolonged ER stress eventually leads to apoptosis the caspase-3/7 activity was determined 24 h after tunicamycin treatment (Fig. 13A). HeLa cells containing HERP-specific siRNAs show a two-fold stronger caspase-3/7 activity compared to those transfected with EGFP pSuper constructs. Thus, HERP can indeed prevent an early enhancement of ER stress-induced caspase-3/7 activity.

To test whether the UBL domain is required for the antiapoptotic function of HERP the same experiment was performed with HeLa cells stably overexpressing full length HERP and a truncated HERP version lacking its UBL domain, called HERP-ΔUBL. Caspase-3/7 acitivity was determined 16, 20 and 24 h after tunicamycin treatment (Fig. 13B). Overexpression of full length HERP had no effect on caspase-3/7 activity compared to wildtype cells. For both wildtype cells and HERP transfectants, only a slight increase of the caspase-3/7 activity over time after tuncamycin treatment was observed. However, the HERP-ΔUBL transfectants exhibited a strong increase in caspase-3/7 activity over time. At 24 h after tunicamycin treatment, activity was twice as high compared to wildtype cells and HERP transfectants. Overexpression of this UBL-deficient HERP version can therefore suppress the antiapoptotic effect of intrinsic full length HERP. This suggests that the UBL domain is indeed required to prevent an early enhancement of ER stress induced apoptosis. As overexpression of full length HERP does not lead to reduced caspase-3/7 levels compared to wildtype HeLa cells it is conceivable that HERP is necessary but not sufficient to rescue from early onset of ER stress-induced apoptosis. Other components such as HRD1 are also required.

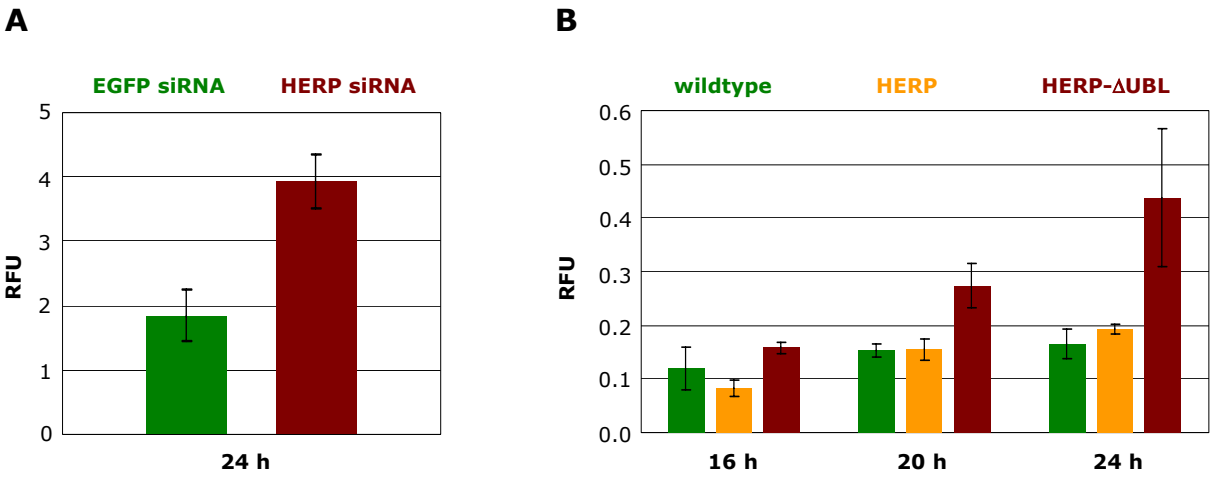

Figure 13. Downregulation of HERP protein levels as well as overexpression of UBL deficient HERP results in an enhancement of ER stress-induced caspase-3/7 activity. HeLa cells were transiently transfected with pSuper constructs encoding *HERP*- or *EGFP*-specific siRNAs **(A)** or stably transfected with constructs driving the expression of *HERP* or *HERP-ΔUBL* **(B)** and treated with 10 µg/mL tunicamycin. Caspase-3/7 activity was determined 24 h after addition of tunicamycin (A) or as indicated below the graph (B) using the Apo-ONE homogenous caspase-3/7 assay (Promega). RFU = relative fluorescent units.

3.2.3 HERP is cleaved by a caspase-3/7 activity upon prolonged ER stress

The above experiments have demonstrated that an siRNA-mediated reduction of HERP expression as well as overexpression of UBL-deficient HERP leads to an enhancement of ER stress-induced caspase-3/7 activity. This suggests that HERP rescues the cell from early onset of ER stress-induced apoptosis. Computational analyses of the HERP primary structure revealed two potential caspase-3/7 cleavage sites (DXXD) in its C-terminal region (Cohen, 1997). The first one, DWLD (263-266), is located at the beginning of the predicted transmembrane region (264-284), the second one, DVLD (355-358), is located closer to the C-terminus. Therefore, prolonged ER stress might lead to HERP cleavage by a caspase-3/7 activity. To address this and to clarify which of the two potential cleavage sites is used for HERP cleavage, HeLa cells were treated with tunicamycin and the caspase-3/7 inhibitor DEVD-FMK. Cells were harvested before treatment and at 8, 24 and 48 h after commencement of the treatment. Western blot analysis of whole cell lysates was performed with a HERP-specific antibody (Fig. 14). In the absence of the caspase-3/7 inhibitor HERP expression was strongly induced within 8 h as expected. At 24 h after tunicamycin addition a second band corresponding to about 55 kDa appeared. This second band was even more prominent in the cells lysed 48 h after tunicamycin addition. In cells, which were treated with the caspase-3/7 inhibitor, the production of this cleavage product was prevented. Only a small portion of the HERP protein was cleaved 48 h after ER stress induction when DEVD-FMK was present. Thus, upon apoptosis HERP is cleaved by a caspase-3/7 activity. The HERP cleavage product has an apparent molecular mass of about 55 kDa compared to the 62 kDa of full length HERP, which indicates that HERP appears to be cleaved at the C-terminal caspase cleavage site DVLD (355-358).

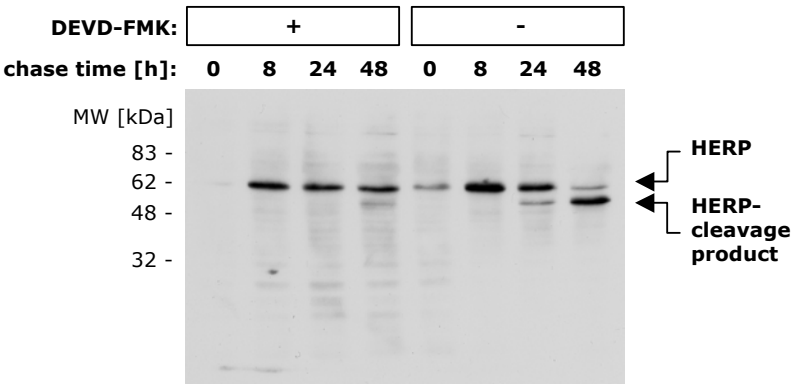

Figure 14. HERP is cleaved by a caspase-3/7 activity. HeLa cells were treated with 10 μg/mL tunicamycin and 10 mM DEVD-FMK, a caspase-3/7 inhibitor, or DMSO as negative control. Cells were harvested at the indicated time points and whole cell lysates were subjected to SDS-PAGE followed by immunoblotting with the anti-HERP antibody.

3.2.4 HERP is essential for the degradation of the ERAD substrate CD3-delta

Proteins that are upregulated by increased synthesis during the UPR pathway either play a role as chaperones to assist protein folding or function in the ERAD pathway to eliminate accumulated proteins from the ER. Considering that HERP is anchored in the ER membrane with both the N- and the C-terminus facing the cytoplasm, where certain steps of the ERAD pathway occur, HERP presumably functions in ERAD. To elucidate this, the turnover of an ERAD model substrate was determined in the absence and presence of HERP-specific siRNAs. As an ERAD substrate the T-cell receptor subunit CD3-delta was chosen, which was shown to be a substrate of the ERAD E3 ligase HRD1 (Kikkert et al., 2004). HeLa cells expressing CD3-delta and siRNAs specific for either EGFP or HERP were treated with cycloheximide to block translation. Degradation of CD3-delta could thereby be monitored by harvesting the cells at various time points after cycloheximide addition and performing Western blot analysis with an antibody specific for CD3-delta. To see whether the proteasome was responsible for CD3-delta degradation the proteasome inhibitor MG132 was added to the cells (Fig. 15A). In the presence of EGFP siRNAs CD3-delta is degraded rapidly with a half life of one hour. In contrast, downregulation of HERP synthesis resulted in a stabilisation of the ERAD substrate. This led to the accumulation and therefore to increased steady state levels of CD3-delta as demonstrated by the band intensities at time point zero. Surprisingly, the proteasome inhibitor MG132 had only a partial effect on CD3-delta stabilisation. Thus, although CD3-delta has been described to be degraded by the proteasome (Yang et al., 1998), there might be other proteases involved in CD3-delta degradation. However, irrespective of the identity of the protease degrading CD3-delta, the HERP protein is essential for its efficient turnover. The densitometric analyses of the band intensities from figure 15A further clarified this effect (Fig. 15B). Western blot analyses demonstrated that the HERP siRNA construct inhibited HERP synthesis when coexpressed with the CD3-delta encoding construct (Fig. 15C).

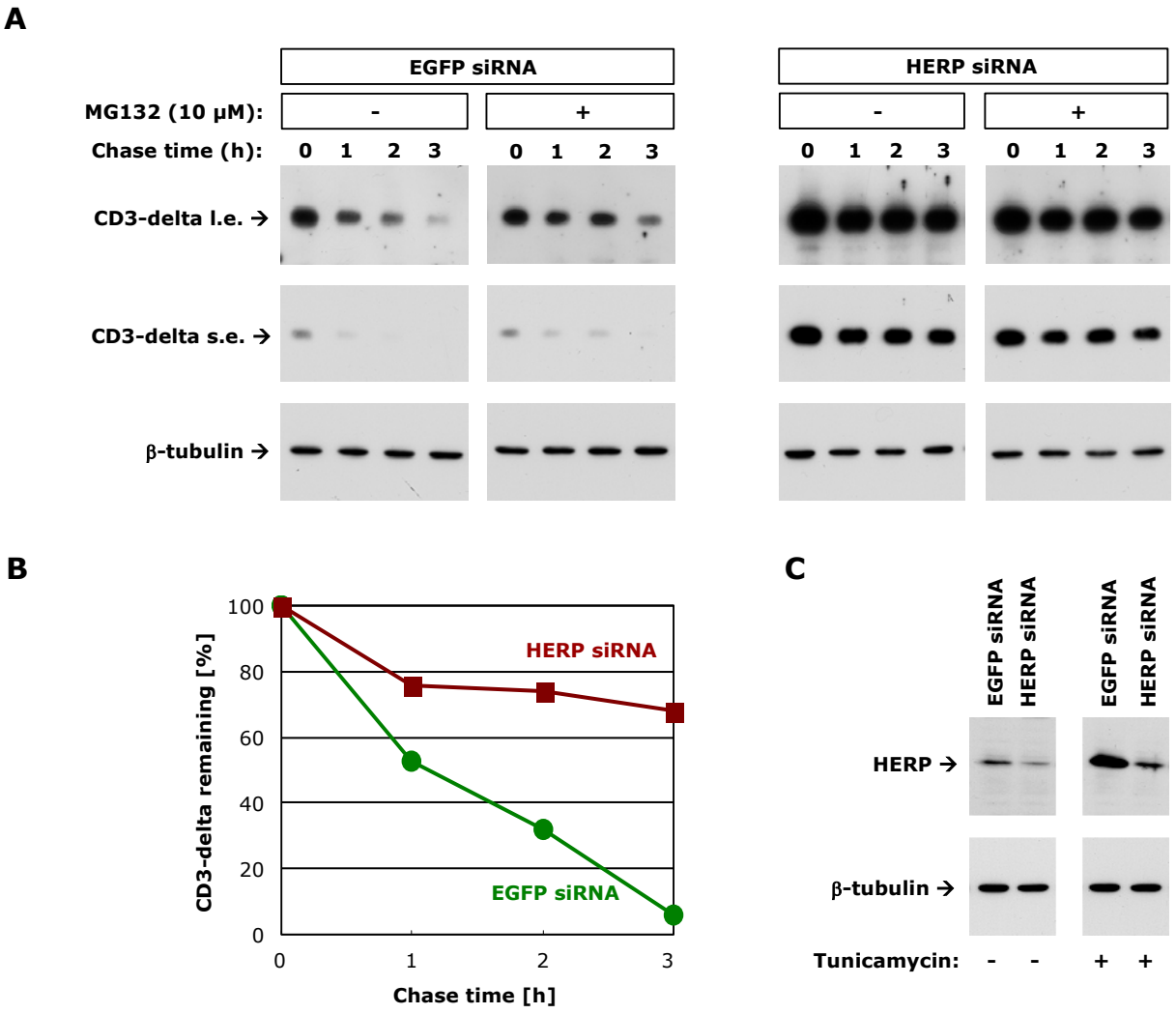

Figure 15. An siRNA-mediated repression of HERP synthesis resulted in stabilisation of the ERAD substrate CD3-delta. (A) HeLa cells were cotransfected with plasmids encoding CD3-delta and pSuper constructs containing sequences for siRNAs specific for either *EGFP* or *HERP*. At 48 h after transfection 50 µg/mL cycloheximide was added to all samples and 10 µM MG132 was supplemented at 0 h. Cells were harvested at the indicated time points and Western blot analysis was performed with antibodies specific for CD3-delta and for β-tubulin. l.e. = long exposure, s.e. short exposure. **(B)** Densitometric analysis of the chase experiments from (A). **(C)** HERP-specific siRNA inhibited HERP protein synthesis when CD3-delta was coexpressed. HeLa cells were transfected as described in (A) and lysed 48 h after transfection. Western blots of the cell lysates were stained with antibodies specific for HERP and for β-tubulin. Prior to cell lysis cells were treated with 10 µg/mL tunicamycin for 4 h where indicated.

Since the UBL domain of HERP is essential for rescuing the cell from ER stress-induced apoptosis, it was speculated that this domain would also be critical for the degradation of ERAD substrates. To address this, the turnover of CD3-delta was determined upon overexpression of full length HERP and UBL-deficient HERP-ΔUBL (Fig. 16). This experiment demonstrated that overexpression of HERP-ΔUBL resulted in a striking stabilisation of the ERAD substrate CD3-

delta. In contrast, overexpression of full length HERP had no effect on CD3-delta degradation compared to mock-transfected cells. This was consistent with the finding that overexpression of HERP could not rescue cells from ER stress-induced apoptosis, while overexpression of UBL-deficient HERP led to a dramatic increase of caspase-3/7 activity. Thus, HERP is not sufficient to eliminate misfolded proteins from the ER and might require other components of the ERAD pathway to efficiently enhance the degradation of ERAD substrates. Similar to the experiments in figure 15, this experiment also demonstrated that CD3-delta degradation was not fully blocked by the addition of the proteasome inhibitor MG132. Taken together, this shows that HERP is essential for the degradation of the model ERAD substrate CD3-delta and that the UBL domain of HERP is required for proper HERP functioning.

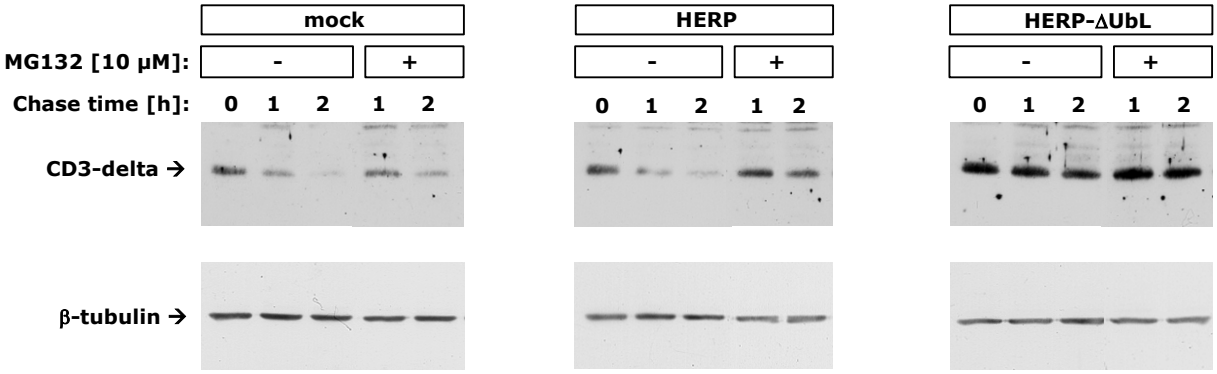

Figure 16. Overexpression of UBL-deficient HERP resulted in the stabilisation of the ERAD substrate CD3-delta. HeLa cells transiently expressing CD3-delta or coexpressing CD3-delta and HERP and CD3-delta and HERP-ΔUBL were treated with 50 μg/mL cycloheximide at time point 0 h to block translation. Where indicated MG132 was added to a final concentration of 10 μM. Cells were harvested at the indicated time points and subjected to Western blot analysis (20 μg lysate/lane) using CD3-delta- and β-tubulin-specific antibodies.

3.2.5 HERP cosediments with other ERAD components

The experiments described so far demonstrated that HERP is essential for the efficient degradation of the model ERAD substrate CD3-delta. Therefore, HERP might interact with other ERAD components such as HRD1, Derlin-1, VIMP, p97 or the proteasome to cooperate with these proteins in eliminating misfolded proteins from the ER. To elucidate whether HERP cosediments with other ERAD components glycerol gradient centrifugation was performed. The gradient was harvested into 13 fractions, which were analysed by Western blot analysis with

antibodies specific for the different ERAD components (Fig. 17). HERP and HRD1 were found to comigrate in the gradient and were detected mainly in the fractions 8, 9 and 10. The ATPase p97 was also found in these fractions although most of it was present in fractions 5 and 6. Derlin-1 was detected in every fraction of the gradient except of fraction 1. However, lower amounts of Derlin-1 were detected in the last three fractions. The proteasome was found in fractions 6 to 10 as shown by staining for the 20S proteasomal subunit β1. Thus, subpopulations of p97, Derlin-1 and the proteasome were present in the same fractions as HERP and HRD1. This holds the possibility that HERP and the other ERAD components might form a common protein complex. As HERP and HRD1 comigrate with the 26S proteasome, this potential high molecular mass complex is at least the size of the 26S proteasome, which has a molecular weight of 2.5 MDa.

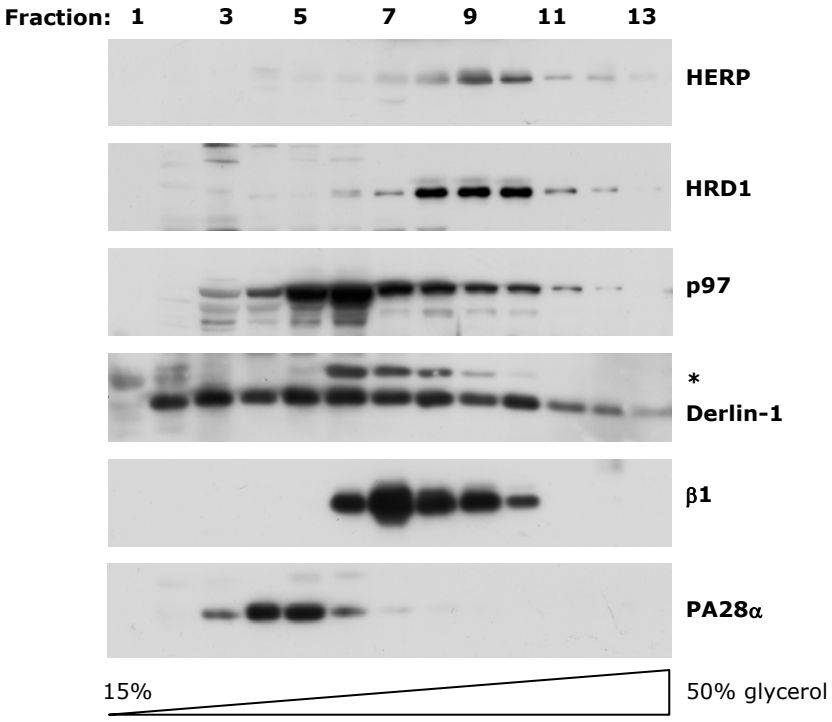

Figure 17. HERP cosediments with other ERAD components. Ramos cells (3 x 10^7) were lysed and separated on a 15-50% glycerol gradient. Fractions were precipitated with trichloroacetic acid and then solubilised in SDS sample buffer. Western blots of the gradient fractions were performed either with HERP antiserum or with antibodies specific for HRD1, p97, Derlin-1, the proteasomal subunit β1 and the α subunit of the proteasome activator PA28. The asterisk indicates unspecific cross-reaction of the Derlin-1 antibody, as verified by the deduced molecular mass.

3.2.6 The UBL domains of HERP and HHR23B display different binding properties towards the proteasome

Many UDPs have been described to interact with the 26S proteasome and this interaction was shown to be dependent on the UBL domains of these proteins (Funakoshi et al., 2002; Schauber et al., 1998). As HERP contains a UBL domain it might also have the capacity to bind to the

proteasome. To address this question, HERP and HHR23B were expressed as fusion proteins containing a Protein A ZZ tag in HeLa cells. HHR23B, which interacts with the proteasome via its UBL domain (Schauber et al., 1998), served as a positive control. Cell lysates of the transfectants were subjected to immunoprecipitation analysis using IgG Sepharose and proteins, which were coprecipitated with the ZZ fusion proteins, were analysed via SDS-PAGE and immunodetection with antibodies specific for the 20S proteasomal subunit β1 (Fig. 18A, middle panel) and the 19S subunit S1 (Fig. 18A, lower panel). As expected, the ZZ fusion of HHR23B precipitated the proteasome. Surprisingly, the HERP protein did not share this proteasome binding property of HHR23B and other members of the UDP family. To exclude the possibility that the failure of the HERP UBL domain to bind the proteasome is due to the non-UBL part of the protein, which might mask the region responsible for interaction, a hybrid version of HERP was designed in which the UBL domain was replaced by the UBL domain of HHR23B. Immunoprecipitation analyses of this HERP-hybrid fusion revealed an interaction with the proteasome, even though this interaction was weaker than that of wildtype HHR23B. Thus, the different binding properties of HERP compared to other UDPs such as HHR23B are apparently due to structural differences within the UBL domain.

This experiment was confirmed by *in vitro* pull-down analyses with GST-tagged HHR23B and different GST-tagged versions of HERP as well as the HERP-hybrid. To enable an efficient expression in *E. coli* cells the HERP fusion proteins were expressed without their C-terminus containing the transmembrane helices. After expression of the GST fusion proteins, they were immobilised on glutathione Sepharose and incubated with either HeLa lysates or *E. coli* lysates containing recombinant S5a. S5a is the proteasomal subunit responsible for the interaction with HHR23B (Hiyama et al., 1999). Western blots of the pull-down experiments demonstrated that GST-tagged HHR23B and the HERP-hybrid precipitated the proteasome from HeLa lysates as shown for the 19S proteasomal subunit S1 (Fig. 18B, middle panel). These fusion proteins could also precipitate recombinant S5a from *E. coli* lysates (Fig. 18B, lower panel). In contrast, the truncated HERP versions HERP-ΔC, HERP-ΔC-ΔUBL and the UBL domain of HERP, HERP-UBL, did not interact with the proteasome from HeLa lysates. However, when incubated with *E. coli* lysates containing recombinant S5a a very weak interaction with this overexpressed recombinant protein was detected. This interaction was indeed UBL-dependent since the UBL-deficient version of HERP could not precipitate recombinant S5a. However, as the binding capacity of HERP to S5a is at least two orders of magnitude weaker compared to HHR23B, the functional relevance of this interaction is questionable. Thus, HERP does not share the

proteasome binding property with HHR23B and other members of the UDP family. The aberrant binding capacity is solely dependent on structural differences within the HERP UBL domain. This suggests that HERP is not capable of recruiting the proteasome to the site of protein degradation at the ER and that it therefore has another role within ERAD.

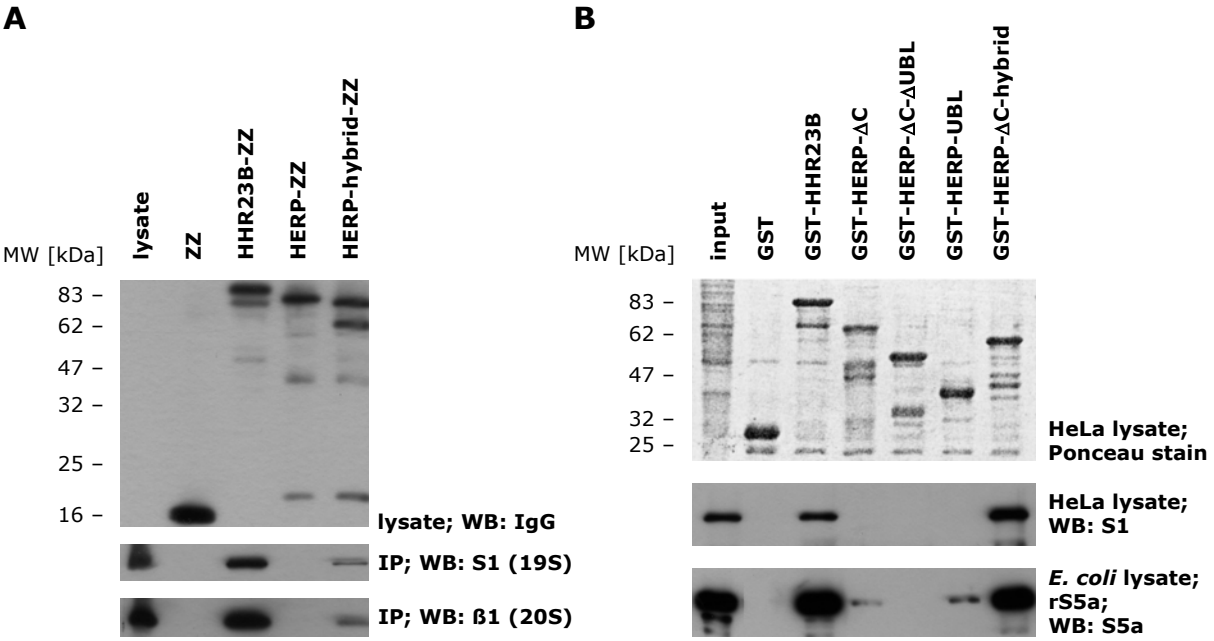

Figure 18. The UBL domains of HERP and HHR23B display different binding properties towards the proteasome. (A) HeLa cells transiently expressing ZZ or ZZ fusion proteins of HHR23B, HERP and the HERP-Hybrid were lysed and subjected to immunoprecipitation with IgG Sepharose. For a depiction of the fusion constructs please refer to the appendix. The fusion proteins were cleaved with TEV protease to release the proteins and their binding partners into the supernatant. The upper panel shows a Western blot of the lysates of the different transfectants incubated with rabbit IgG to visualise equal expression of the ZZ fusions. After SDS-PAGE the samples were visualised using specific antibodies against the base subunit S1 (middle panel) and the 20S subunit β1 (lower panel). **(B)** GST or GST fusion proteins from HHR23B, HERP-ΔC, HERP-ΔC-ΔUBL, HERP-UBL or the HERP-ΔC-Hybrid immobilised on glutathione Sepharose were used to precipitate either the proteasome complex from HeLa cell lysate (middle panel) or the recombinant proteasome subunit S5a from *E. coli* lysate (lower panel). The precipitates were analysed by Western blot analysis with antibodies specific for the proteasomal subunits S1 or S5a. The upper panel shows the Western blot membrane stained with Ponceau S to visualise the fusion proteins bound to glutathione Sepharose. IP = immunoprecipitation, WB = Western blot.

3.2.7 HERP interacts with ERAD components

To elucidate whether HERP interacts with other ERAD components that act upstream of the proteasome, immunoprecipitations were performed with Protein A ZZ or ZZ fusions of HERP, the truncated version of HERP lacking its UBL domain and HHR23B. HeLa lysates containing these fusion constructs were incubated with IgG Sepharose and Western blots of the

immunoprecipitates were analysed using antibodies specific for p97, Derlin-1, VIMP, the proteasome subunits S1 and β1 and calnexin. To study the HRD1 interactions, cells coexpressing myc-tagged HRD1 and the different ZZ fusions were used (Fig. 19C). The HERP full length and HERP-ΔUBL fusion proteins were found to interact with overexpressed HRD1-myc and with intrinsic p97, Derlin-1 and VIMP, while non of the HERP fusions could precipitate the proteasome. HHR23B, on the other hand, did not bind to any of the ERAD components tested, but efficiently precipitated the proteasome. Thus, HERP interacts directly or indirectly with the ERAD components HRD1, p97, Derlin-1 and VIMP. This interaction is not dependent on the HERP UBL domain, since UBL-deficient HERP is also capable of binding these ERAD components.

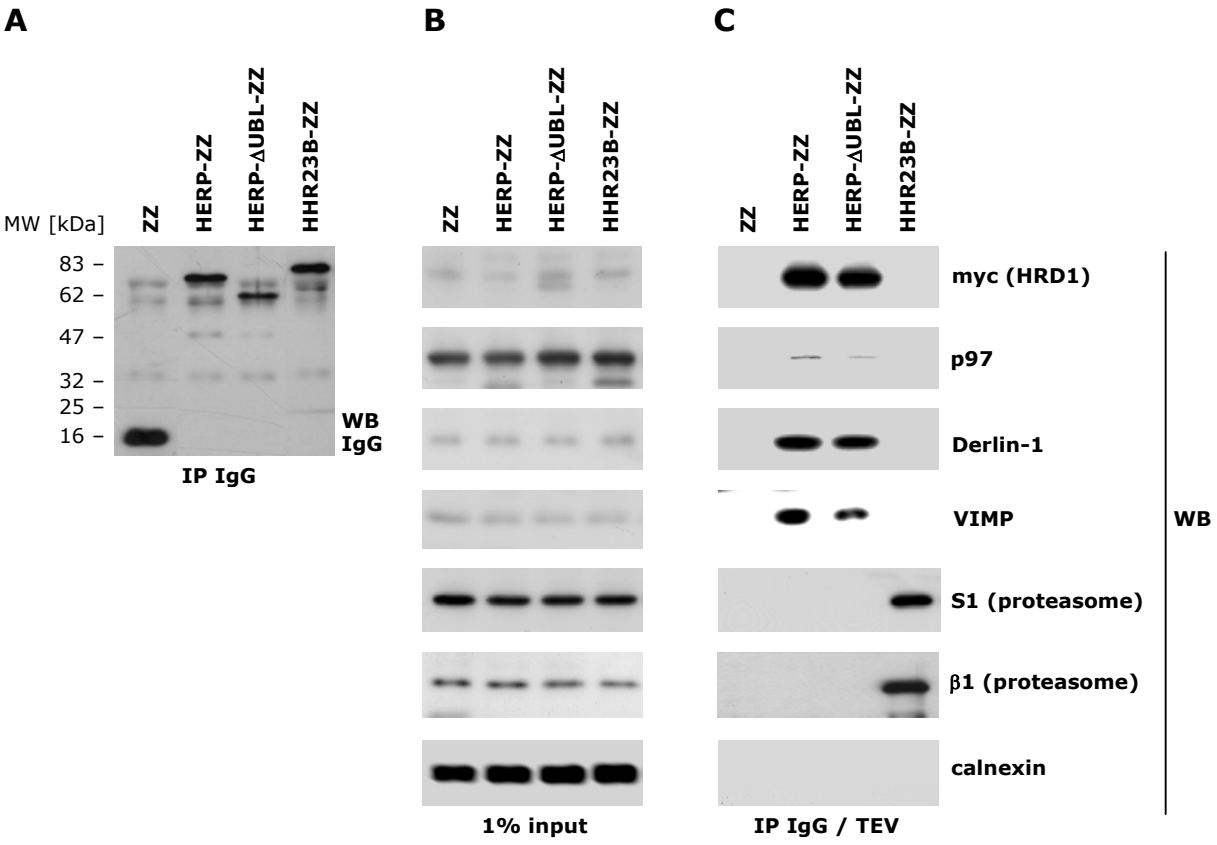

Figure 19. HERP interacts with ERAD components. HeLa cells (4 x 10^6) were transfected with plasmids encoding ZZ, HERP-ZZ (aa 1-391), HERP-ΔUBL-ZZ (aa 88-391) and HHR23B-ZZ (aa 1-409). For a depiction of the fusion constructs please refer to the appendix. At 24 h after transfection cells were lysed and extracts were incubated with IgG Sepharose (IP IgG) for 2 h. For coprecipitation studies with HRD1 cells coexpressing myc-tagged HRD1 and the different ZZ fusions were used. After extensive washing, the beads were cleaved with TEV protease to release the proteins into the supernatant, which was then separated via SDS-PAGE. **(A)** Western blots of the immunoprecipitations prior to TEV cleavage were stained with rabbit preserum to show that comparable amounts of ZZ fusions were precipitated. Western blots of input lysates **(B)** and of precipitated proteins released from the IgG beads **(C)** were incubated with antibodies specific for the indicated proteins. IP = immunoprecipitation, WB = Western blot.

These experiments demonstrated that HERP interacts with all ERAD components tested such as HRD1, p97, Derlin-1 and VIMP. Taking into account that these proteins also comigrate in glycerol gradients it is feasible that they all are components of a multisubunit protein complex. To test this possibility, microsomal membranes were prepared from Ramos cells, solubilised and used for immunoprecipitation with antibodies against the 20S subunit α6, HERP, HRD1, p97, VIMP and rabbit preserum as a negative control. Western blots of the immunoprecipitates were incubated with antibodies against p97, HRD1, HERP, Derlin-1, VIMP and the proteasomal subunit β1. As the Derlin-1 antibody does not recognise Derlin-1 when in complex with other ERAD components (Ye et al., 2005), an immunoprecipitation with this antibody was not performed. Western blots of the immunoprecipitates demonstrated that p97 and Derlin-1 could be coprecipitated with HERP, HRD1, p97 and VIMP, but not with the proteasome (Fig. 20). Therefore, the proteins p97 and Derlin-1 can bind all ERAD components tested. HRD1 and HERP were precipitated with the anti-HERP, the anti-HRD1 and the anti-p97 antibodies. Surprisingly, an interaction of HERP and HRD1 with VIMP could not be detected, although there was a clear interaction in the immunoprecipitation analyses with the ZZ-tagged HERP versions (Fig. 19).

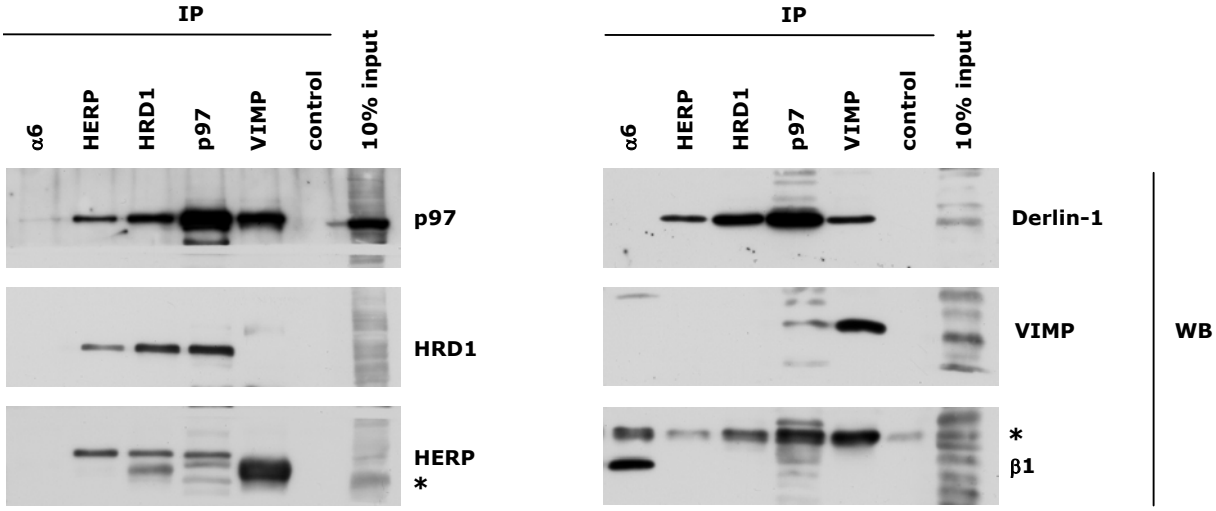

Figure 20. HERP, HRD1, p97 and Derlin-1 form a protein complex. Microsomal membranes were prepared from Ramos cells, solubilised and used for immunoprecipitation with antibodies specific for the proteasomal subunit α6, HERP, HRD1, p97 and VIMP. Preimmune serum was used as a control. The immunoprecipitates were analysed by Western blotting with antibodies specific for p97, HRD1, HERP, Derlin-1, VIMP and the proteasomal subunit β1. The asterisks indicate IgG heavy chain (for the HERP blot) and IgG light chain (for the β1 blot), which crossreact with Protein A-HRP. IP = immunoprecipitation, WB = Western blot.

Since HRD1 and HERP were also not detected in the 10% input lanes with the VIMP antibody, the HRD1-VIMP and HERP-VIMP interactions might be below detection level in this case. However, the antibodies for HERP and HRD1 were also not capable to precipitate VIMP, while the VIMP antibody could detect a band in the 10% input lane. Therefore, the interaction of VIMP with HRD1 and HERP may indeed be questioned and two different complexes might exist, one containing HERP, HRD1, p97 and Derlin-1 and the other one consisting of p97, Derlin-1 and VIMP.

This experiment was repeated in collaboration with Yihong Ye from Boston who used solubilised dog pancreas microsomes for immunoprecipitation with antibodies against HERP, HRD1, p97, VIMP and rabbit preserum as a negative control. Immunoblots of these precipitates demonstrated that HERP and HRD1 can be detected in the immunoprecipitates with antibodies specific for HERP, HRD1, p97 and VIMP (Fig. 21). Thus, in this experiment VIMP appeared to be present in the same complex as HERP and HRD1. Unfortunately, a Western blot analysis with the VIMP-specific antibody was not performed. The two experiments in figures 20 and 21 were also performed with different antibodies directed against HRD1 and p97. This combined with the different microsomes used makes it difficult to compare the two results. Derlin-1 was also found to be precipitated with antibodies against HERP, HRD1, p97 and VIMP. The same is true for p97, although its interaction with the HRD1 antibody was very weak. However, the anti-p97 antibody efficiently precipitated HRD1, suggesting that the HRD1 antibody most likely competed with p97 for HRD1 binding. This would explain the weak p97 band detected in the HRD1 precipitate.

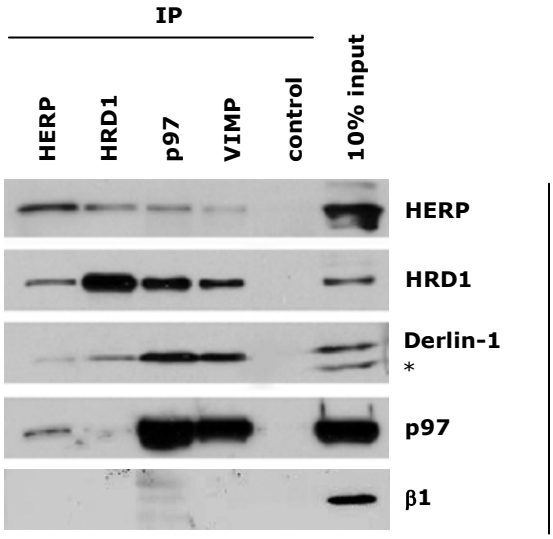

Figure 21. HERP, HRD1, p97, Derlin-1 and VIMP form a protein complex. Solubilised dog pancreas microsomes were used for immunoprecipitation with antibodies specific for HERP, HRD1, p97 and VIMP. Preimmune serum served as control. The immunoprecipitates were analysed by Western blotting with antibodies specific for HERP, HRD1, p97, Derlin-1 and the proteasomal subunit β1. The asterisk indicates a cross-reaction of the Derlin-1 antibody. IP = immuno-precipitation, WB = Western blot.

Taken together, these data on the intrinsic interactions of different ERAD components (Fig. 20 and 21) support the data obtained from the glycerol gradients (Fig. 18) and the immuno-precipitations with ZZ-tagged proteins (Fig. 19), and indicate that HERP, HRD1, p97 and Derlin-1 form a common protein complex. The question whether VIMP is also part of this protein complex or whether it forms a second complex with p97 and Derlin-1 could not be answered yet.

To further substanciate the notion that the ERAD components form a protein complex, a glycerol gradient was performed and each fraction was precipitated with an antibody specific for p97. Immunoblots of these precipitates showed that HRD1 was precipitated from the fractions 8 to 12 with the p97 antibody (Fig. 22). In the first gradient experiment (Fig. 17) these were also the fractions in which HRD1 and HERP were detected. Derlin-1 was also precipitated from these fractions (Fig. 22). Accordingly, although Derlin-1 is present in every fraction of the glycerol gradient (Fig. 17), it only interacts with p97 when HRD1 and HERP are present. Therefore, HRD1, Derlin-1 and p97 are present in a common protein complex. Unfortunately, it was difficult to detect the HERP protein in this experiment. It migrates to almost the same position as IgG heavy chain from the immunoprecipitation, which is also detected by the secondary antibody. However, as shown in figure 17 HERP is only present in those fractions of the glycerol gradient, in which HRD1 and Derlin-1 can be coprecipitated with p97 (Fig. 22). Therefore, this clearly demonstrates that HERP, HRD1, p97 and Derlin-1 form a protein complex at the ER membrane.

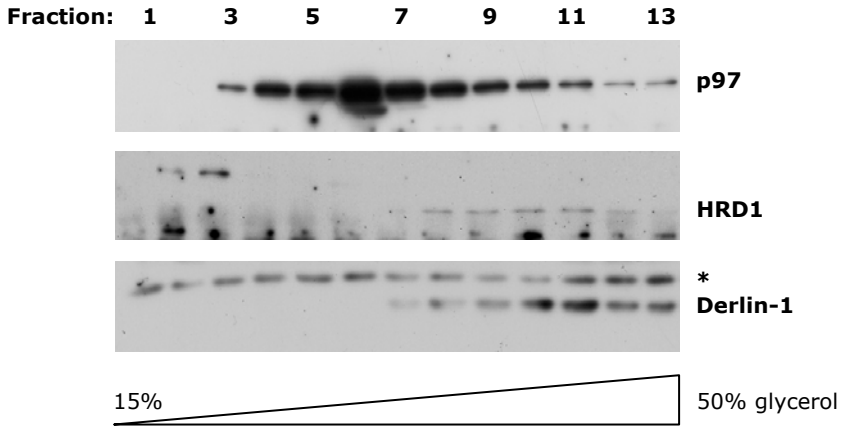

Figure 22. ERAD components that cosediment in a glycerol gradient form a protein complex. Ramos cells (3 x 10^7) were lysed and separated on a 15-50% glycerol gradient. Gradient fractions were incubated with an anti-p97 antibody and Protein G Sepharose. After washing the beads were resuspended in SDS sample buffer. Western blots of the gradient fractions were performed with antibodies specific for HRD1, p97 and Derlin-1. The asterisk indicates signals corresponding to IgG light chain of the p97 antibody, which cross-reacts with the Derlin-1 antibody.

To get insight into the direct interactions of the complex, different truncated versions of HERP as well as the C-terminal cytosolic regions (Ccr) of HRD1 and Derlin-1 were expressed in *E. coli* as GST fusions and immobilised on glutathione Sepharose. The beads containing the different GST fusions were incubated with *E. coli* lysate containing either His-tagged HRD1-Ccr or His-tagged p97. As shown in figure 23, GST-tagged HERP-ΔC and HERP-ΔC-ΔUBL directly interact with His-HRD1-Ccr, but not with His-p97. The UBL domain of HERP bound neither His-tagged HRD1-Ccr nor His-p97. Thus, as demonstrated in figure 19 the direct interaction between HERP and HRD1 is independent of the HERP UBL domain. GST-tagged HRD1-Ccr, on the other hand, bound to both His-HRD1-Ccr and His-p97. The same was true for the His-tagged C-terminal cytosolic region of Derlin-1.

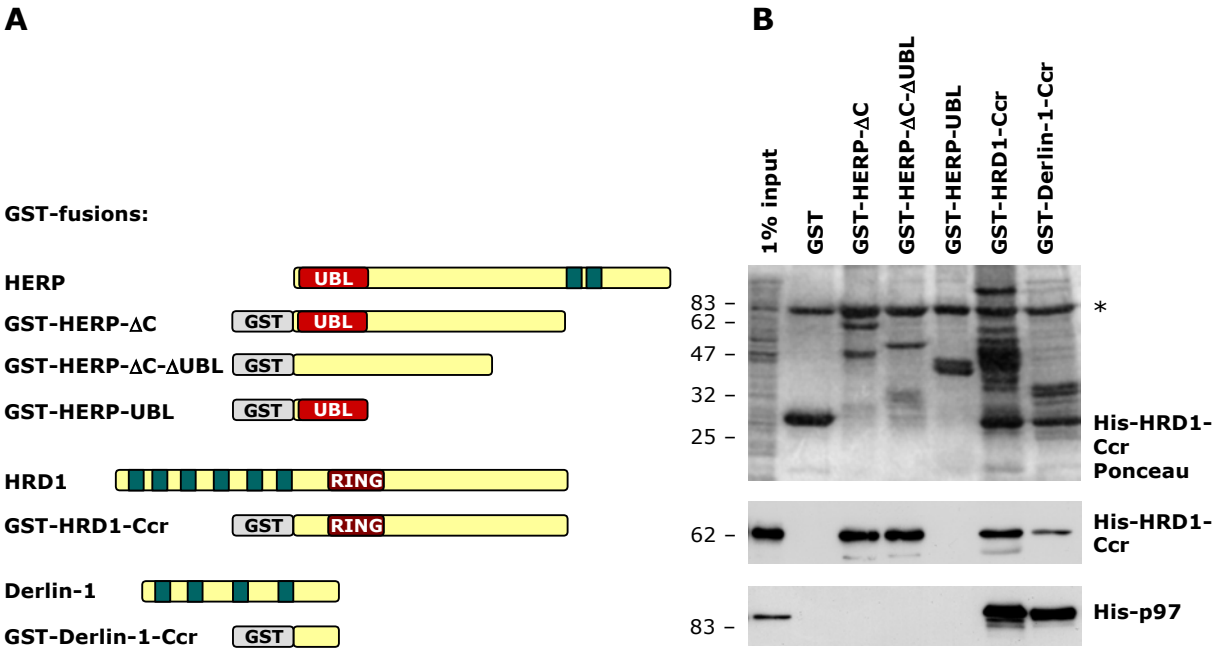

Figure 23. Direct interactions within the ERAD complex. GST or GST fusions of HERP-ΔC (aa 1-240), HERP-ΔC-ΔUBL (aa 88-240), HERP-UBL (aa 1-87), the C-terminal cytosolic regions of HRD1 (HRD1-Ccr, aa 236-616) and Derlin-1 (Derlin-1-Ccr, aa 176-251) were expressed in *E. coli*, immobilised on glutathione Sepharose and incubated with lysates from *E. coli* containing either His-tagged HRD1-Ccr or His-tagged p97. **(A)** Schematic representation of the different GST fusions used compared to the full length versions. Dark blue boxes represent transmembrane helices. **(B)** Western blots of the pull-down experiments. The upper panel shows a Western blot membrane of the pull-downs with His-tagged HRD1-Ccr stained with Ponceau S to visualise loading of the GSH beads. The asterisk (*) marks BSA, which was included in the buffer. Western blots of the pull-downs performed with His-HRD1-Ccr and His-p97 incubated with an anti-His-antibody are shown in the middle and in the lower panel, respectively. Positions of standard protein markers are shown on the left with their sizes in kDa.

Thus, HERP directly interacts with the E3 ligase HRD1, whereas HRD1 interacts with various components of the complex. This study revealed direct interactions of HRD1 to HERP, p97 and Derlin-1. Moreover, since GST-HRD1-Ccr was found to interact with His-tagged HRD1-Ccr, the formation of HRD1 dimers or even oligomers is possible. Derlin-1 also binds directly to p97 as well as to HRD-1. Thus, similar to HRD1 this protein might have a central position within the ERAD complex.

3.2.8 HERP is stabilised upon overexpression of the E3 ligase HRD1

The experiments presented so far demonstrated that HERP and HRD1 always comigrate in glycerol gradients and that they directly interact with each other. This indicated that they form a functional unit and that their association is a prerequisite for the activity within the ERAD complex. To test whether the E3 ligase HRD1 has an impact on the stability of HERP a cycloheximide chase was performed with HeLa cells expressing either myc-tagged HRD1 or the mutated HRD1(C329S). The mutant version of HRD1, HRD1(C329S), is not functional since it lacks an essential cysteine residue of the RING domain responsible for coordinating a zinc ion. In cells transfected with an empty myc vector HERP was degraded rapidly with a half life of about 1.5 to 2 h (Fig. 24A). Four h after cycloheximide addition less than 10% of the original amount of HERP protein were detected. In contrast, overexpression of HRD1-myc led to a stabilisation of the HERP protein, resulting in a half life of approximately 8 h. This effect was independent of the E3 ligase activity of HRD1 since overexpression of the HRD1 mutant HRD1(C329S) also resulted in a stabilisation of HERP. Presumably, HRD1 stabilises HERP by binding to it. The degradation of HERP was dependent on the 26S proteasome, as was shown by the addition of the proteasome inhibitor MG132, which prevented the degradation of HERP (Fig. 24).

HERP was shown to interact with other E3 ligases such as the RING E3 ligase RBX1, a component of the SCF complex (data not shown). To test whether this protein can also stabilise the HERP protein, the same experiment was performed with HeLa cells overexpressing ZZ-tagged RBX1. As shown in figure 24B, overexpression of this protein had no effect on the stability of HERP. Since RBX1 is a component of an SCF complex, it might not be able to stabilise HERP when not being complexed with a cullin and other SCF components.

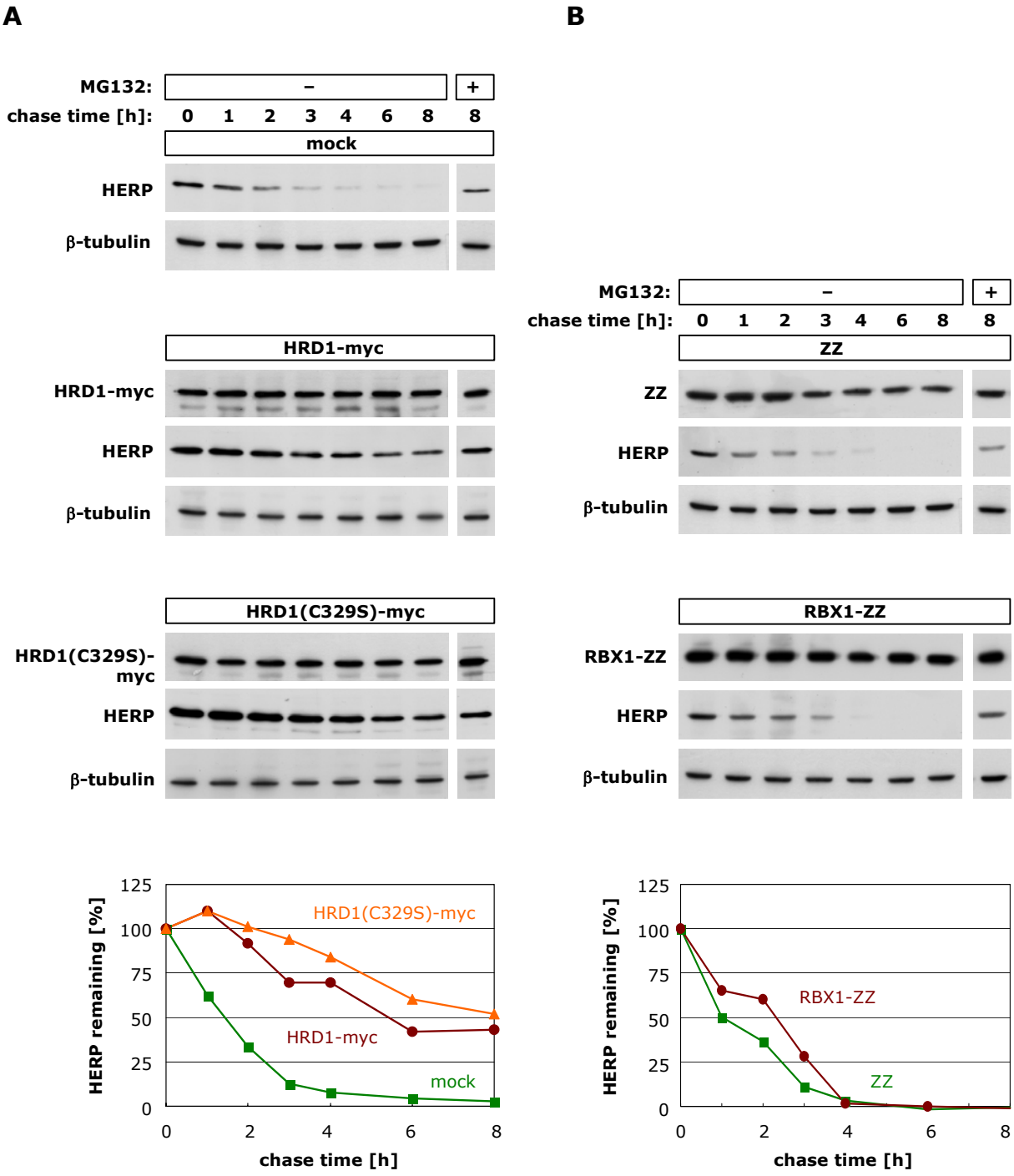

Figure 24. Overexpression of the E3 ligase HRD1 leads to a stabilisation of HERP. (A) HeLa cells were either transfected with empty pCMV-Tag 3B vector (mock) or with a plasmid encoding HRD1-myc or the mutant version HRD1-C329S-myc. At 20 h after transfection the cells were treated with 10 µg/ml tunicamycin and 24 h after transfection cycloheximide was added to inhibit translation. The proteasome inhibitor MG132 was added when indicated. Cells were harvested and lysed at the indicated time points after cycloheximide addition and subjected to Western blot analysis using antibodies against HERP, myc and β-tubulin. **(B)** The same experiment was performed with HeLa cells expressing ZZ-tagged RBX1 or ZZ only as a control.

The SCF component RBX1 is a cytosolic protein, which does not contain transmembrane helices. Transmembrane proteins, however, especially those containing a tag, tend to form aggregates when overexpressed in the cell. Thus, it is feasible that overexpressed HRD1-myc and HRD1(C329S)-myc bind to HERP and accumulate in the cell forming HERP-HRD1-myc aggregates. To check for such aggregates glycerol gradients were performed with lysates from HeLa cells transfected with HRD1-myc or HRD1-(C329S)-myc (Fig. 25). In the gradients of mock-transfected cells HERP was present in the fractions 8 to 12 and some p97 and Derlin-1 was also detected in these fractions. However, compared to figure 17 less of p97 and Derlin-1 comigrated with HERP. This might be due to the different cell types used for the different experiments. Ramos cells, which were used for the gradient in figure 17, secrete IgM and therefore contain large amounts of ER. Accordingly, their steady state levels of HRD1 and HERP synthesis are higher compared to that of HeLa cells (data not shown) and more p97 and Derlin-1 are in complex with HERP and HRD1. In glycerol gradients from cells containing HRD1-myc HERP clearly comigrated with the overexpressed E3 ligase (Fig. 25). Interestingly, in addition to the peak at fractions 9 to 11, a second peak appeared at fractions 5 to 7 in which both proteins were also detected. Since p97 was most prominent in these fractions, this indicated that these HRD1-HERP-complexes were associated with p97 in these fractions. However, figure 22 clearly indicated that p97 from those slowly migrating fractions was not associated with HRD1 and Derlin-1. Thus, HERP from these fractions was probably associated with one or a few HRD1 proteins, forming a complex of about 600 kDa, the size of the p97 hexamer. Basically, the same is true for the glycerol gradient of HeLa cells overexpressing HRD1(C329S)-myc. Although a second peak was not detected, mutant HRD1 and HERP comigrated very strinkingly and portions were also found in fractions of lower density. Therefore, overexpressed wildtype and mutant HRD1 are assembled into the ERAD complex and are also present in a slower migrating protein complex also containing HERP.

In addition to this observation, larger amounts of Derlin-1 and p97 seemed to sediment in fractions of higher density in the gradients with overexpressed HRD1-myc and HRD1(C329S)-myc compared to mock-transfected cells (Fig. 25). This indicated that wildtype and mutant HRD1 might associate with other monomeric ERAD components such as p97 and Derlin-1 leading to the formation of more ERAD complexes.

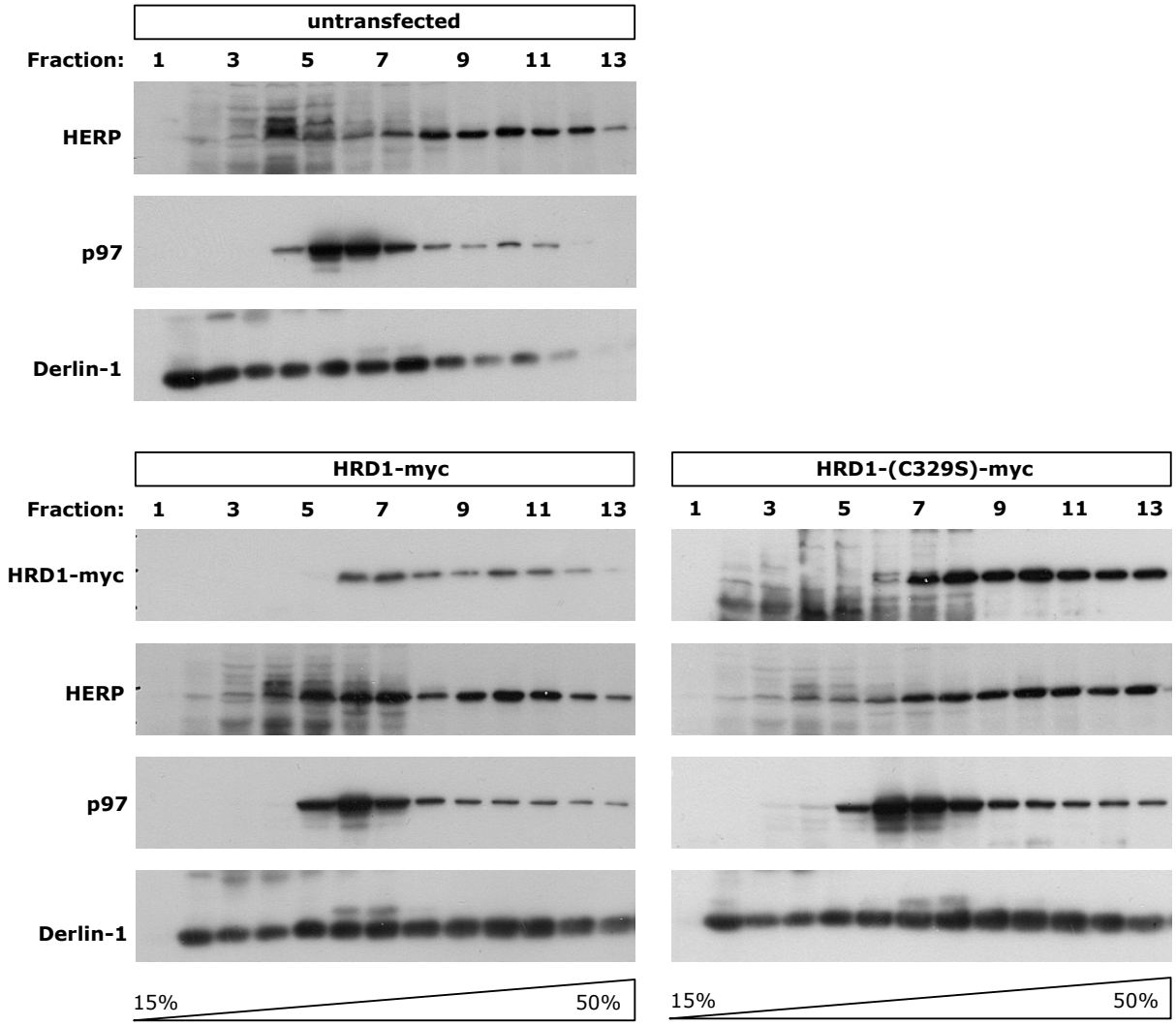

Figure 25. Overexpressed HRD1-myc and HRD1(C329S)-myc associate with ERAD components resulting in an enhanced formation of ERAD complexes. HeLa cells were transfected with constructs encoding HRD1-myc or HRD1(C329S)-myc. At 48 h after transfection cells were lysed and separated on a 15-50% glycerol gradient. Fractions were precipitated with trichloroacetic acid and then solubilised in SDS sample buffer. Western blots of the gradient fractions were performed with HERP antiserum or with antibodies specific for HRD1, p97 or Derlin-1.

Since HERP is upregulated in HeLa cells upon induction of the UPR, it is conceivable that HERP stability is altered during ER stress, when it is required for the survival of the cell. To address this, cycloheximide chase experiments were performed with untreated HeLa cells and with HeLa cells in which the UPR was induced by the addition of tunicamycin 4 h prior to the addition of cycloheximide (Fig. 26). Tunicamycin addition led to an upregulation of HERP synthesis as reflected by the increased amount of HERP at time point 0 in lysates of tunicamycin-

treated cells compared to untreated cells. However, upon ER stress the turnover rates of HERP were not altered. In untreated cells and in cells treated with tunicamycin HERP had a half life of about 2 h. Thus, in contrast to overexpression of the E3 ligase HRD1, induction of the UPR did not result in a stabilisation of HERP.

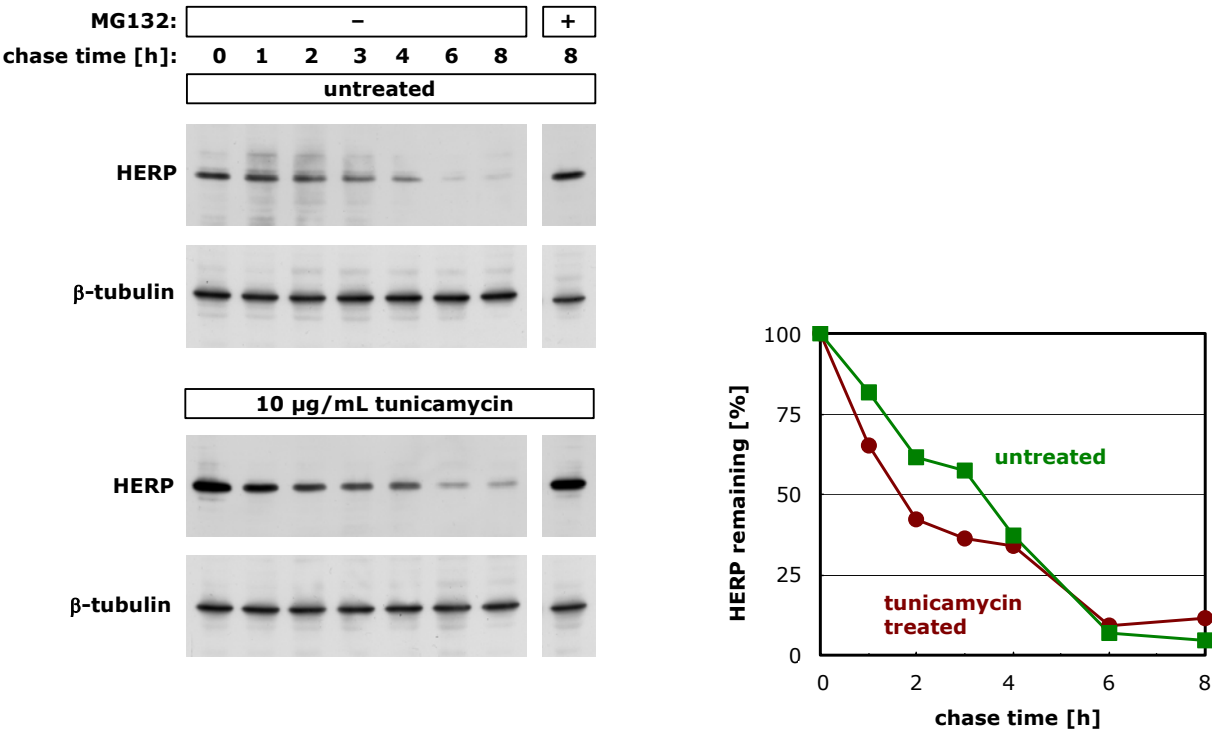

Figure 26. Stability of HERP with and without ER stress. HeLa cells were left untreated or were treated with 10 µg/mL tunicamycin for 4 h. Then, cycloheximide was added to a final concentration of 50 µg/mL (time point 0) to block translation. Cells were harvested and lysed at the indicated time points after cycloheximide addition and subjected to Western blot analysis using antibodies specific for HERP or β-tubulin. Where indicated, the proteasome inhibitor MG132 was added to a final concentration of 10 µM at time point 0 of the chase. The graph on the right shows a densitometric analysis of the chase experiments.

3.2.9 USP7 - a target for the UBL domain of HERP

So far, it was demonstrated that HERP interacts directly with HRD1 and that these two proteins form a protein complex with p97, Derlin-1 and possibly VIMP. However, the direct interaction of HERP with HRD1 was shown to be independent of the HERP UBL domain. In addition, the UBL domain of HERP was shown not to share the proteasome binding properties of other UDPs. Thus, an interaction partner for the HERP UBL domain has not been identified yet. However, Madelon Maurice from Utrecht identified HERP in a yeast two-hybrid screen to search for novel interaction partners of the deubiquitylating enzyme USP7. Thus, HERP and the DUB USP7 might also interact with each other.

USP7 is a large protein of 1,100 amino acids and a molecular weight of 129 kDa. It contains a poly-glutamine stretch and a MATH (meprin and TRAF homology) domain at the N-terminus as well as a UCH domain for deubiquitylating activity and a coiled coil domain (Fig. 27). The N-terminus with the poly-glutamine stretch and the MATH domain were found to interact with HERP in the yeast two hybrid screen.

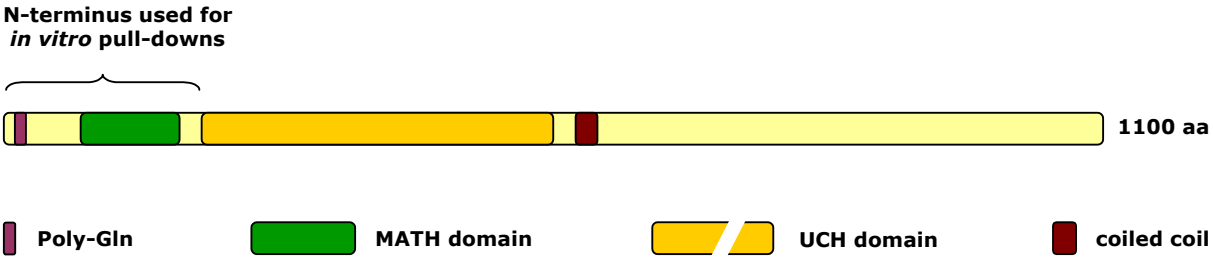

Figure 27. Domain architecture of USP7. Schematic representation of the domain architecture of USP7. The different domains are colour-coded according to the key below. The N-terminal region of USP7 containing the poly-glutamine stretch and the MATH domain, which was found to interact with HERP in the yeast two-hybrid sceen and which was used for the *in vitro* pull-down experiments, is marked.

To test whether USP7 indeed binds to HERP and to identify the HERP region responsible for USP7 interaction, immunoprecipitation analyses were performed with ZZ fusions of HERP, HERP-ΔUBL, HHR23B and another UDP called Ubiquilin-1 as well as the UBL deficient version of Ubiquilin-1. HeLa cell lysates containing these fusion proteins were incubated with IgG Sepharose. Proteins that coprecipiated with the ZZ fusions were analysed by Western blot analysis with antibodies specific for USP7 (Fig. 28C, upper panel) and the proteasomal subunit S5a as a control (Fig. 28C, lower panel). This experiment clearly demonstrated that HERP can

interact with the deubiquitylating enzyme USP7, whereas there was no interaction of USP7 with the other UDPs Ubiquilin-1 and HHR23B. Interestingly, this interaction is UBL-dependent since the UBL-deficient version of HERP did not precipitate USP7. As a positive control the Western blots were also incubated with an antibody specific for the proteasomal subunit S5a demonstrating that ubiquilin-1 and HHR23B interact with S5a in a UBL-dependent manner (Fig. 28C, lower panel).

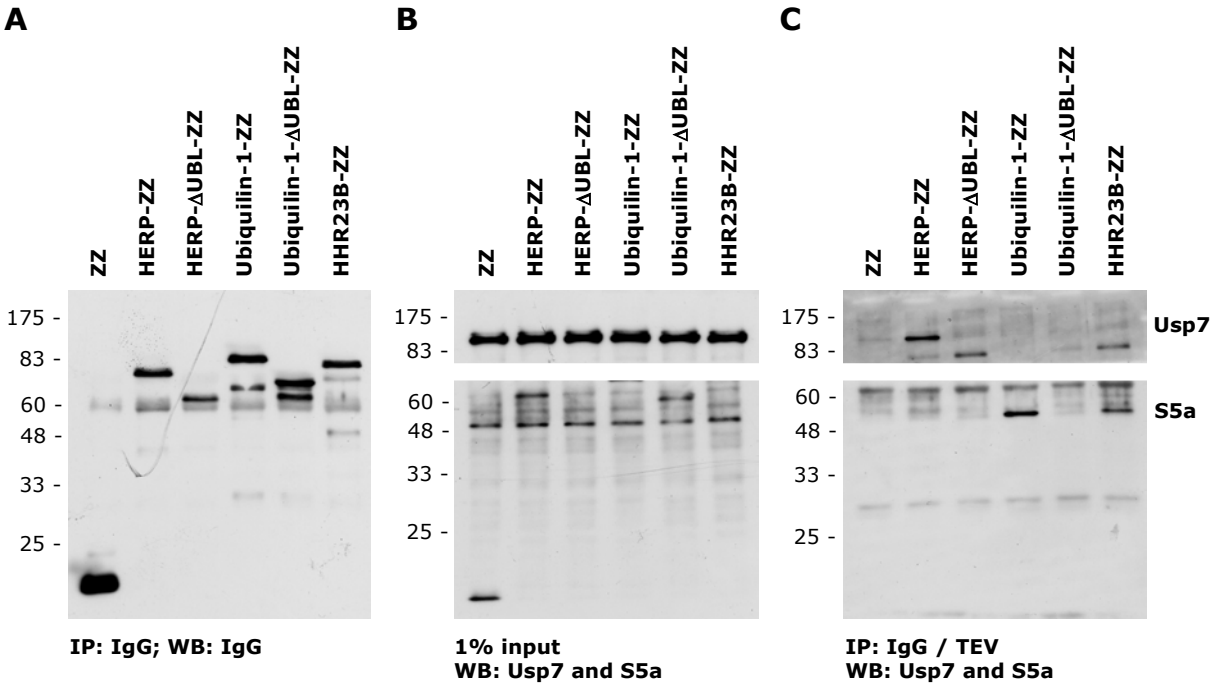

Figure 28. HERP interacts with USP7. HeLa cells (4 x 10^6) were transfected with plasmids encoding ZZ, HERP-ZZ (aa 1-391), HERP-ΔUBL-ZZ (aa 88-391), Ubiquilin-1-ZZ (aa 1-589), Ubiquilin-1-ΔUBL-ZZ (aa 112-589) and HHR23B-ZZ (aa 1-409). For a depiction of the fusion constructs please refer to the appendix. At 24 h after transfection cells were lysed and extracts were incubated with IgG-Sepharose (IP IgG) for 2 h. After extensive washing the beads were cleaved with TEV protease to release the proteins into the supernatant, which was then separated via SDS-PAGE. **(A)** A Western blot of the immunoprecipitations prior to TEV cleavage was stained with rabbit preserum to show that comparable amounts of ZZ-fusions were precipitated. Western blots of the input lysates **(B)** as well as precipitated proteins released from the IgG beads **(C)** were incubated with antibodies specific for USP7 (upper panels) and S5a (lower panels). Positions of standard protein markers are shown on the left with their sizes in kDa.

To further characterise the interaction between HERP and USP7, *in vitro* pull-down experiments were performed with GST-tagged versions of HERP-ΔC, HERP-ΔC-ΔUBL, HERP-UBL and HHR23B. These fusion proteins were expressed in *E. coli* and immobilised on glutathion

Sepharose. The beads loaded with the fusion proteins were incubated with lysates from Ramos cells and Western blots of these pull-down experiments were incubated with antibodies specific for USP7 and S5a. As shown in figure 29A HERP and the UBL domain of HERP efficiently precipitated USP7 from Ramos cells while there was no interaction of USP7 with the UBL-deficient HERP version. Thus, the interaction of HERP with USP7 indeed depends on the HERP UBL domain.

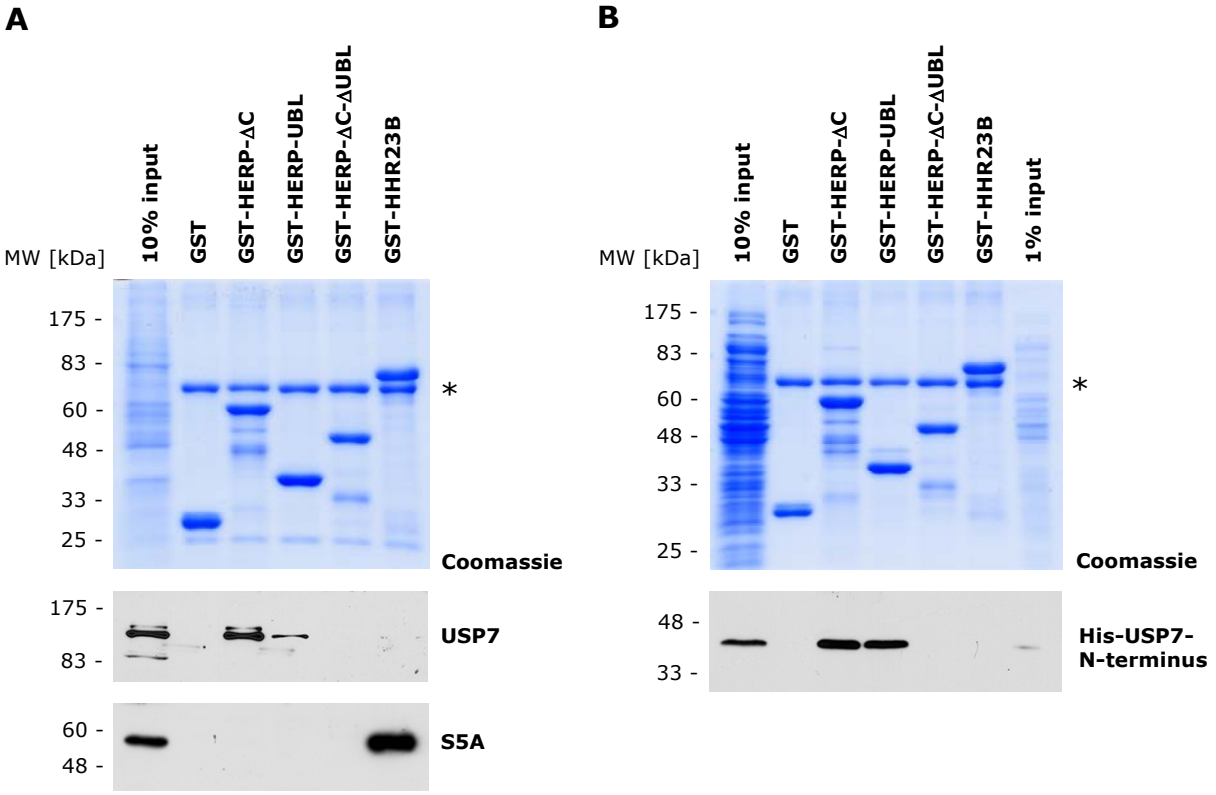

Figure 29. Interaction between HERP and USP7. GST and GST fusions of HERP-ΔC (aa 1-240), HERP-ΔC-ΔUBL (aa 88-240), HERP-UBL (aa 1-87) and HHR23B (aa 1-401) were expressed in *E. coli*, immobilised on glutathione Sepharose and incubated either with lysates from Ramos cells **(A)** or with lysates from *E. coli* containing the N-terminal region of USP7 **(B)**. For a depiction of the fusion constructs please refer to the appendix. The upper panels show a Coomassie-stained acrylamide gel of the pull-downs after SDS-PAGE to visualise loading of the glutathione beads. The asterisks (*) mark BSA which was included in the buffer. Western blots of the pull-downs performed with Ramos lysates were incubated with antibodies against USP7 and S5a **(A)** while Western blots of the pull-down experiments with His-tagged USP7 N-terminus were performed with an anti-His antibody **(B)**.

To test whether these proteins can directly interact with each other a His-tagged version of the N-terminus of USP7 (Fig. 27) was generated and expressed in *E. coli* cells. This N-terminus of USP7 containing a poly-glutamine stretch and a MATH domain was found to interact with HERP in the yeast two-hybrid screen. Therefore, this region was also chosen for the *in vitro* pull-down. The different GST fusion proteins bound to glutathione Sepharose were incubated with *E. coli* lysates containing the His-tagged N-terminus of USP7 (Fig. 29B). Western blot analysis of these pull-down experiments showed that HERP as well as the UBL domain of HERP could precipitate the N-terminus of USP7. Therefore, HERP directly interacts with the N-terminal region of the DUB USP7 containing a poly-glutamine region and the MATH domain.

To elucidate the functional relevance of the interaction between HERP and USP7 and to clarify whether USP7 also has a role in ERAD, three USP7-specific siRNAs were tested for their efficiency in downregulating USP7 synthesis. HeLa cells were transfected with one or a combination of two of these USP7-specific siRNAs and the silencing efficiency was monitored by Western blot analysis (Fig. 30). The downregulation of USP7 was very efficient with all siRNAs tested and the transfection with either one was sufficient for downregulating USP7 synthesis. Functional analyses to study the effect of USP7 on the stability of the ERAD substrate CD3-delta or the effect of USP7 on the stability of ERAD components such as HERP or HRD1 are now under way.

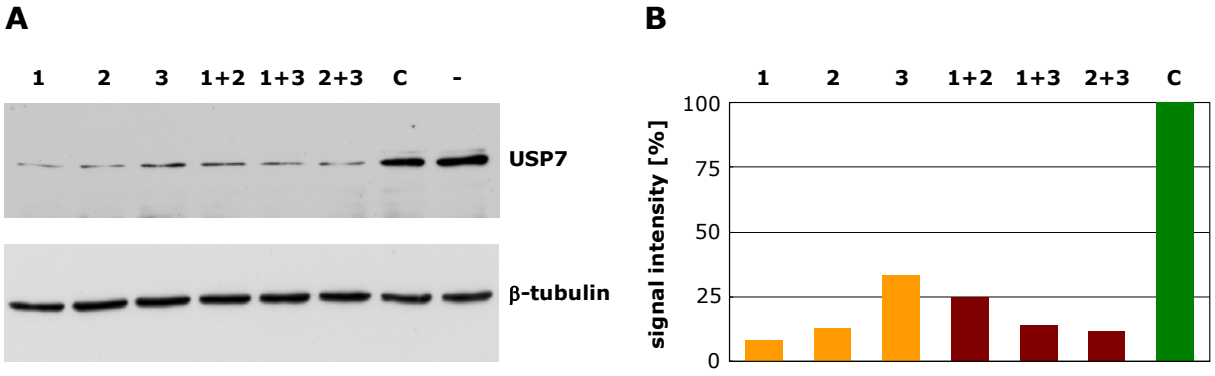

Figure 30. Downregulation of USP7 synthesis by different USP7-specific siRNAs. (A) HeLa cells were transfected with the USP7-specific siRNAs 1, 2 and 3 (see table 10 in section 2.3.5 for sequences) and with combinations of two siRNAs as well as a control siRNA. At 24 h after transfection the cells were harvested and lysed with RIPA buffer. Cell lysates (20 µg) were subjected to SDS-PAGE and Western blot analysis with antibodies specific for USP7 or β-tubulin. C = control. **(B)** Densitometric analysis of the Western blot from (A). The signal intensity of the control (green) was defined as 100% to compare the silencing efficiency of the different siRNAs (orange) and combinations of two of those (dark red).

4 DISCUSSION

4.1 A role for HERP within the UPR

4.1.1 HERP rescues the cell from ER stress-induced apoptosis

The ER resident protein HERP has been identified in a screen searching for genes that are upregulated in human umbilical vein endothelial cells (HUVEC) treated with homocysteine (Kokame, 1996; Kokame et al., 2000). Interestingly, this compound was shown to induce ER stress, since certain ER stress-inducible proteins, e.g. BIP, GRP94, PDI and calnexin, were upregulated upon treatment of cells with homocysteine (Kokame et al., 2000). Thus, HERP was shown to be an ER stress-inducible protein, which in addition to homocysteine is also induced by classical ER stress inducers such as tunicamycin and thapsigargin (Kokame et al., 2000). The *HERP* promoter contains ER stress response elements (ERSE), which are present in the promoters of various UPR target genes (van Laar et al., 2000). However, van Laar and coworkers showed that synthesis of HERP (designated as MIF1 in that study) was upregulated not only by ER stress, but also by osmotic stress and by the DNA-damaging, alkylating agent methylmethanosulfonate. This observation was supported by the finding that besides the ERSE elements the HERP promoter contains a so-called ATF-C/EBP composite site (Ma and Hendershot, 2004). This site is the target of ATF-4, which is activated by several cellular stressors. Thus, the activity of HERP might be required for the cell during various stress conditions.

Although the regulation of *HERP* expression has been studied in detail, the role of HERP within the UPR was unknown so far. Certain proteins with a role in the UPR including BIP, HRD1 and Parkin were shown to protect cells from apoptosis after induction of ER stress (Imai et al., 2000; Kaneko et al., 2002; Sugawara et al., 1993). The findings presented here demonstrate that the ER resident protein HERP also prevents early onset of ER stress induced apoptosis. Partial inhibition of *HERP* expression by RNA interference resulted in an enhancement of caspase-3/7 activity after induction of apoptosis by tunicamycin. In addition, the expression of a UBL-deficient version of HERP led to an increased vulnerability to ER stress-induced apoptosis. Thus, the UBL domain of HERP plays an important role in preventing ER stress-induced apoptosis and might therefore be responsible for recruiting other factors to the ERAD complex that are required for the efficient degradation of misfolded proteins from the

ER. In this study an interaction of the HERP UBL domain with the DUB USP7 was found, suggesting that USP7 might be involved in ERAD. The observation that overexpression of the full length version of *HERP* had no effect on caspase-3/7 activity indicates that HERP is necessary but not sufficient to protect the cell from ER stress-induced apoptosis. Other factors that act in close cooperation with HERP such as HRD1 might also be required. The antiapoptotic capacity of HERP was recently demonstrated by two other groups as well. Hori and coworkers created a HERP-deficient F9 embryonic carcinoma cell line and found that these cells were more vulnerable to ER stress compared to F9 control cells (Hori et al., 2004). This effect was rescued by the expression of *HERP* cDNA in HERP-deficient cells, but not by the expression of an N-terminally truncated version of HERP lacking its UBL domain. In another study, Chan and coworkers demonstrated that suppression of HERP by RNA interference sensitises neuronal cells to apoptosis induced by ER stress (Chan et al., 2004). They also demonstrated that PC12 cells overexpressing wildtype *HERP* were significantly more resistant to death induced by ER stress. While the first finding is in line with the data presented here, the second result was not verified in my study. This might be due to the different cell types used. Overall, the results from these labs are consistent with the observations presented here and support the idea that HERP is essential to rescue the cell from ER stress-induced apoptosis. In addition, the HERP UBL domain was shown to be required for efficient ERAD.

The present study also revealed that HERP is cleaved by a caspase-3/7 activity upon ER stres-induced apoptosis. A 50 kDa fragment of HERP was produced during apoptosis, suggesting that the C-terminal caspase cleavage site (DVLD355) is used. Consequently, a short C-terminal fragment with strong hydrophobicity (Fig. 6) is released into the cytoplasm. Although this study did not address possible cytotoxic properties of the released HERP fragment it is likely that this hydrophobic fragment forms cytotoxic aggregates in the cytosol enforcing apoptosis. Another possibility would be that the C-terminus of HERP is also required for the interaction with an essential ERAD compontent. Accordingly, the antiapoptotic function of HERP might be compromised under prolonged ER stress, thereby further supporting apoptosis. Consistent with these results, Chan and coworkers also demonstrated that HERP is cleaved in PC12 cells during ER stress-induced apoptosis. However, they found that HERP was cut at both caspase-3/7 cleavage sites (Chan et al., 2004). This discrepancy might be due to the different cell types or to the different concentrations of tunicamycin used in both studies. HERP cleavage at DWLD266 releases an N-terminal fragment of about 30 kDa containing the UBL domain into the cytosol. Since the UBL domain is required to protect the cell from ER stress-induced apoptosis, it is likely

that the cleavage of HERP at DWLD266 reduces its intrinsic antiapoptotic function at the ER. In this regard, it is noteworthy that the N-terminal UBL domain of parkin, which also confers cytoprotection towards ER stress (Imai et al., 2000), is similarly cleaved and removed by caspases (Kahns et al., 2002).

4.1.2 The cytoprotective effects of HERP are due to its essential role in ERAD

Proteins that are induced by the UPR, like HERP, either play a role as chaperones to assist in refolding events of misfolded ER proteins, or function in ERAD for the disposal of misfolded proteins from the ER. As HERP contains a UBL domain, which indicates a role in the ubiquitin-proteasome system, and most of the HERP protein is exposed to the cytoplasmic side of the ER membrane, where important steps of the ERAD pathway occur, it was more likely that HERP has a role in ERAD. This hypothesis was tested and it was demonstrated that HERP is required for the degradation of the model ERAD substrate CD3-delta. Similar to results from the caspase-3/7 cleavage assays overexpression of full length HERP had no effect on CD3-delta degradation, whereas expression of UBL-deficient HERP led to a stabilisation of CD3-delta. Consistent with the results presented here connexin-43, an endogenous ERAD substrate (VanSlyke and Musil, 2002), was shown to be stabilised in HERP knockout cells (Hori et al., 2004). This suggests that HERP is indeed required for the efficient elimination of misfolded proteins from the ER and that the UBL domain plays a crucial role in this process. Its important role in ERAD can thereby be considered to underlie its cytoprotective capacity during ER stress.

Interestingly, addition of the proteasome inhibitor MG132 did not result in a complete inhibition of CD3-delta degradation, although HERP itself was efficiently stabilised under the same conditions (compare Fig. 15 with Fig. 24 or 26). This observation appeared to contradict earlier results, where CD3-delta was described to be degraded entirely by the proteasome (Yang et al., 1998). How can this discrepancy be explained? The initial experiments by Yang and coworkers (Yang et al., 1998) were performed in T-lymphocytes, which continually synthesise and degrade subunits of the T-cell receptor (TCR) (Bonifacino et al., 1989). They also used a T-cell line characterised by its failure to express TCR-β. Accordingly, TCRs do not assemble and CD3-delta is degraded (Chen et al., 1988). The authors claimed that the proteasome inhibitor LLnL also prolonged the half-life of CD3-delta when expressed transiently in COS7 cells, although the corresponding data were not shown (Bonifacino et al., 1989). In another study, demonstrating that the E2 MmUBC7 is essential for TCR-α and CD3-delta degradation in HEK-293 cells, they only showed that the proteasome inhibitor lactacystine enhanced the steady state

levels of CD3-delta without performing chase experiments (Tiwari and Weissman, 2001). The following experiments performed in non-T-cells expressing CD3-delta very often did not show a control with proteasome inhibitor (Fang et al., 2001; Zhong et al., 2004). When proteasome inhibitors were added in a chase experiment these had only a minor effect on CD3-delta stabilisation (Kikkert et al., 2004; R. J. Wojcikiewicz, personal communication). Thus, when overexpressed in non-T-cells CD3-delta might not solely be degraded by the proteasome. Other proteases might be involved in the degradation of CD3-delta from non-T-cells as well. Alternatively, CD3-delta might be exported from the cells by exocytosis. Unfortunately, an attempt to identify the alternative proteolytic system responsible for CD3-delta degradation by using inhibitors of various proteases was unsuccessful. Only a partial inhibition of CD3-delta degradation was observed in the samples containing proteasome inhibitors (data not shown). Therefore, the alternative non-proteasomal process by which CD3-delta is eliminated from non-T-cells remains to be identified.

4.2 Novel insights into the role of UBL domains

4.2.1 The UBL domain of HERP does not share the proteasome binding properties of other UDPs

Many UDPs were shown to interact with the proteasome via their UBL domains. Therefore, proteasome binding appeared to be a general feature of all UBL domains. Hence, with regard to the findings that HERP has a role in ERAD it was tempting to specultate that HERP interacts with the proteasome and recruits it to the site of ERAD. Surprisingly however, this is not the case. Immunoprecipitation analyses as well as *in vitro* pull-down assays revealed that, in contrast to HHR23B, which precipitated the proteasome very efficiently in both assays, HERP does not bind the 26S proteasome. Solely by performing an *in vitro* pull-down assay with *E. coli* lysate containing recombinant S5a a very weak interaction of GST-tagged HERP with S5a was observed. This interaction was indeed UBL-dependent, since the UBL-deficient HERP version did not precipitate S5a from *E. coli* lysates. However, the interaction of recombinant S5a with HERP was at least two orders of magnitude weaker compared to the one found with HHR23B. Therefore, the functional relevance of this interaction is questionable. To exclude the possibility that this aberrant binding property towards the proteasome is due to regions within the HERP protein, which might mask the interaction-relevant part of the HERP UBL domain, a hybrid version of

HERP was constructed. This HERP-hybrid contained the UBL domain of HHR23B instead of the HERP UBL domain. The HERP-hybrid bound the proteasome very efficiently in both immunoprecipitation analyses as well as in *in vitro* pull-downs, demonstrating that the different binding properties compared to other UBL domains are indeed due to structural differences within the HERP UBL domain. Thus, HERP is the first UDP described that does not interact with the proteasome.

What is the difference between the HERP UBL domain and other proteasome-binding UBL domains? The primary sequences do not allow to discriminate between the proteasome-binding UBL domains and the HERP UBL domain, since the similarity of the many UBL domains is rather weak. It is noteworthy here that mapping the exact UBL interaction sites within the 26S proteasome revealed that different UDPs apparently bind to different sites within the complex. For example, the human Rad23p homologue HHR23A was shown to associate with the 19S subunit S5a and the interacting region was mapped to the C-terminal of the two ubiquitin-interacting motifs (UIMs) (Hiyama et al., 1999). The yeast Rad23p, on the other hand, interacts with the base subunit Rpn1p and not with the S5a homologue Rpn10p (Elsasser et al., 2002; Saeki et al., 2002; Seeger et al., 2003). Crosslinking experiments also suggested an interaction of Rad23p with Rpn2p, the second non-ATPase subunit of the proteasome base complex (Saeki et al., 2002). These observations were explained by the fact that the C-terminal UIM is not present in Rpn10p. However, although the interaction of yeast Rad23p with the proteasomal subunit Rpn1p is now without controversy, different binding sites within Rpn1p were identified in budding and in fission yeast. In budding yeast a leucine-rich repeat-like domain (LRR-like) was found to be the minimum binding site for the Rad23p UBL domain, whereas in fission yeast another region further N-terminally of the protein was identified (Elsasser et al., 2002; Seeger et al., 2003). Overall, the data suggest that there are significant structural differences within the UBL domains of human HHR23A and its orthologues in fission and budding yeast. Different UDPs possess UBL domains that all seem to bind the proteasome but apparently have different proteasomal targets.

Interestingly, the UBL domain of parkin also differs from other UBL domains in its binding capacity to the proteasome. NMR data have revealed an interaction between the parkin UBL domain and the proteasome subunit S5a. Furthermore, it was demonstrated that this interaction was disrupted in the pathogenic parkin mutant R42P (Sakata et al., 2003). However, in an *in vitro* pull-down assay a physical interaction between the parkin UBL domain and S5a could not be verified, although HHR23A, which was used as a positive control, clearly interacted with

S5a under the same experimental conditions (Sakata et al., 2003). Thus, most likely, the excess of parkin in the NMR studies resulted in detection of an interaction. This interaction, however, was rather weak compared to other UDPs. Therefore, the functional relevance of this interaction between parkin and the proteasome may also be questioned. NUB1 is another protein containing a UBL domain, which is incapable of binding the proteasome. Although, NUB1 was shown to interact with the proteasomal subunit S5a (Kamitani et al., 2001), this interaction was mapped to the C-terminal region of NUB1 and not to the UBL domain (Tanji et al., 2005). Overall this demonstrates that certain UBL domains are not capable of interacting with the proteasome. These proteins might therefore have other cellular targets.

4.2.2 The HERP UBL domain is able to bind USP7

This study has revealed that HERP is able to interact directly with USP7. A UBL-deficient version of HERP did not interact with USP7, demonstrating that this interaction is dependent on the UBL domain of HERP. Thus, the HERP UBL domain, which is incapable of binding the proteasome, has indeed another cellular target. The UBL domains of HHR23B and Ubiquilin-1 do not share this binding capacity to USP7 accentuating the structural differences within the UBL domains of HERP and other UDPs. The UBL domain of HERP was shown to bind the N-terminus of USP7 containing poly-glutamine repeats and a MATH domain. It would be interesting to further narrow down the exact binding site of USP7 to clarify, whether the MATH domain is responsible for this interaction.

During the past years several novel, non-proteasomal UBL targets have been described. This indicates that UBL domains are functionally more diverse than initially thought. One example is the E4 enzyme Ufd2p, which was shown to associate with the UBL domain of Rad23p and to compete with the proteasome subunit Rpn1p for binding Rad23p (Kim et al., 2004). Similarly, the UBL domain of KPC2 was shown to bind the E3 ligase KPC1 and KPC1 competes with the proteasome for binding to the UBL domain of KPC2 (Hara et al., 2005; Kamura et al., 2004). Thus, UBL/UBA proteins appear to directly recruit proteasomal substrates from the site of ubiquitylation to the site of degradation. In another study, the UBL domains of HHR23A and HHR23B were shown to interact with ataxin-3, a protein responsible for the neurodegenerative Machado-Joseph disease (Wang et al., 2000). Since ataxin-3 contains UIMs and since HHR23B was shown to bind the C-terminal UIM of S5a (Hiyama et al., 1999), it was obvious to assume that ataxin-3 interacts with the HHR23B UBL domain in a UIM-dependent manner. However, detailed studies of the interaction demonstrated that the UBL domain of

HHR23B interacts with the ataxin-3 josephin domain, a papain-like cysteine protease similar to other deubiquitylating enzymes (Nicastro et al., 2005; Wang et al., 2000).

Another example is the UBL domain of ubiquilin-1, which was recently reported to interact with Eps15, an essential component of the clathrin-mediated endocytic pathway. Eps15, like S5a, contains two UIMs, but in contrast to S5a ubiquilin-1 was shown to bind the N-terminal one (Regan-Klapisz et al., 2005). Remarkably and in constrast to ubiquilin-1, HHR23B does not bind to Eps15, although both of them interact with the proteasome (data not shown).

Although some UDPs described above do not directly interact with the protasome they all have a role in the ubiquitin-proteasome system. However, a number of UDPs do not display features connecting them to the ubiquitin pathway, indicating that their UBL domains most likely have other non-proteasomal targets. MUBL4, for instance, was recently described to be involved in liver regeneration after partial hepatectomy (Della Fazia et al., 2005). In hepatocytes it was shown to be exported from the nucleus to the cytoplasm during liver regeneration. Therefore, the protein was named HOPS for hematode odd protein shuttling. In contrast, in my experiments with HeLa cells MUBL4 localised to the ER. HOPS was also shown to interfere with protein synthesis by binding to EF-1A, suggesting that it is not involved in the ubiquitin-proteasome pathway and that its UBL domain might have other targets. Another example is the UDP OASL (Fig. 4), a member of the 2´-5´-oligoadenylate synthetases (OAS). These proteins are responsible for the elimination of double-stranded RNA after viral infection. OASL contains two C-terminal UBL domains and these are required for the interaction with MBD1, another protein required for inhibiting viral transcription. Hence, UBL domains can interact with a wide variety of proteins not necessarily linked to the ubiquitin-proteasome pathway. The interactions of different UDPs with the proteasome and with other cellular components are summarised in table 12.

In summary, different UDPs associate with different sites within the 26S proteasome. Moreover, some UDPs do not bind the proteasome at all, while evidence is accumulating that a number of UDPs interact with other proteins, which are not necessarily linked to the ubiquitin-proteasome pathway. The primary structures of the UBL domains that interact with the proteasome (e.g. those of HHR23B and ubiquilin-1) appear as similar to each other as to UDPs like HERP, which do not bind the proteasome (Fig. 8). Therefore, it seems fair to speculate that the ubiquitin superfold provides a basic interaction scaffold, which then aquired substrate specificity during evolution. Proteasome binding by different UBL domains might therefore be rather based on analogous than homologous structures. Thus, UDPs display diverse binding features linking them to a variety of cellular functions.

Table 12. Interactions of various UDPs with the proteasome and/or other UBL targets. The protein, host organism and synomyms are given in the left columns, *S.c. Saccharomyces cerevisiae, S.p. Saccharomyces pombe, H.s. Homo sapiens*. Interactions of the UBL domains with the proteasome are summarised in the middle column, + indicates an interaction, - a reported lacking interaction, ND not determined. The exact interaction site within the proteasome is mentioned, if available. Other UBL targets are given in the right column. For references see main text.

Name	Synonyms	UBL-proteasome interaction		Other UBL targets
S.c. **Rad23p**		+	LRR-like repeat of Rpn1	Ufd2
S.p. **Rhp23**		+	N-term. region of Mts4/Rpn1	
H.s. **HR23A**		+	UIM2 of S5A	Josephin-domain of Ataxin3
H.s. **HR23B**		+		Ataxin3, UFD2
S.c.. **Dsk2p**		+	Rpn1	
S.p. **Dph1**		+	Rpn1	
H.s. **Ubiquilin-1**	hPLIC1	+	S5a	UIM1 of Eps15
H.s. **Ubiquilin-2**	hPLIC2; Chap1	+		
H.s. **Ubiquilin-3**		ND		
H.s. **Ubiquilin-4**	A1Up	+	S5a	
H.s. **NUB1**		-	UIM of S5A, but not via UBL	
H.s. **KPC2**		+		KPC1
H.s. **BAG1**		+		
H.s. **BAG6**	BAT3; Scythe	ND		
S.c. **Ubp6**		+	Rpn1	
H.s. **Usp14**		ND		
H.s. **Parkin**		-		Parkin substrates
H.s. **ElonginB**		ND		
H.s. **HERP**		-		USP7
H.s. **OASL**		ND		MBD1

4.3 A role for HERP in ERAD

4.3.1 A novel protein complex that permits the integration of ubiquitylation and retrotranslocation

This study demonstrated that HERP is essential for the degradation of the model ERAD substrate CD3-delta. Thus, HERP might interact with other components of the ERAD pathway. Since HERP does not bind the 26S proteasome, it might interact with other components, that act further upstream within ERAD. To test this hypothesis potential interactions of HERP with the retrotranslocation component p97, the ERAD E3 ligase HRD1 and the recently identified novel

ERAD component Derlin-1 were studied. Interestingly, HERP interacted directly or indirectly with all ERAD components tested. Further interaction studies revealed that HERP is a component of a protein complex, which also includes HRD1, p97 and Derlin-1. This data therefore clearly demonstrated that within ERAD the ubiquitylation machinery is closely associated with components responsible for translocating substrates across the ER membrane and releasing them into the cytosol. Thus, ubiquitylation and retrotranslocation are performed by a common protein complex, which permits the close coordination of these consecutive steps within ERAD.

Experiments on the structural arragements within this ERAD complex revealed that HERP directly interacts with HRD1, while HRD1 can also associate directly with p97 and Derlin-1. Additionally, GST-tagged HRD1 was found to directly interact with His-tagged HRD1. Thus, HRD1 can form dimers or even oligomers, suggesting that one ERAD complex might harbour two or more HRD1 molecules. The direct interactions of ERAD components observed in this study are summarised in figure 31.

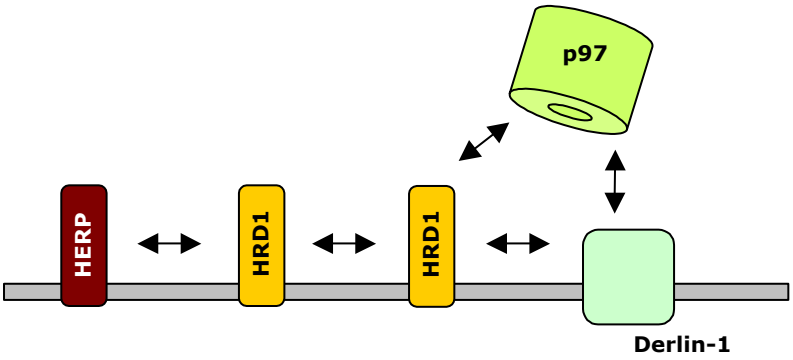

Figure 31. Direct interactions between ERAD components. Schematic drawing of the direct interactions of ERAD components found in this study (black arrows).

These findings extend the previously observed coprecipitation of the ERAD E3 ligase gp78 with p97 from cell extracts (Zhong et al., 2004) by demonstrating that HRD1 can similarly be co-precipitated with p97 and by showing a direct interaction of an ERAD E3 ligase and p97. In this context it is noteworthy that gp78 and HRD1 exhibit considerable similarity in their N-terminal and RING finger regions (Kikkert et al., 2004) and that both E3 ligases aid in the degradation of the ERAD substrate CD3-delta (Fang et al., 2001; Kikkert et al., 2004). Thus, these E3 ligases might cooperate in ubiquitylating at least some ERAD substrates. Since this study demonstrated a direct interaction between two HRD1 molecules, it is tempting to speculate that HRD1 and gp78 can also form heterooligomers and that both E3s can be part of a single protein complex.

The interactions among different ERAD components were recently also mapped by Ye and coworkers (Ye et al., 2005). Their study revealed direct interactions between p97 and Derlin-1 as well as between p97 and HRD1, confirming the results obtained in this study. Additionally, a direct interaction between p97 and gp78 was demonstrated, which is in line with data from Zhong and coworkers (Zhong et al., 2004). However, in contrast to the data shown here, Ye and coworkers did not observe a direct interaction between the E3 ligases gp78 or HRD1 with Derlin-1. This may be explained by the stringent washing conditions they used for their pull-down experiments, thereby making it difficult to detect the interaction of Derlin-1 with VIMP and p97. Since in my experiments the HRD1-Derlin-1 interaction was also much weaker compared to the other direct interactions shown, the interaction of ERAD E3 ligases with Derlin-1 is presumably below detection level in the study by Ye and coworkers. Taken together the data presented here are consistent with results from other groups and demonstrate that the ubiquitylation machinery directly associates with components involved in retrotranslocation of ER-derived substrates to facilitate ERAD.

The study presented here also addressed the potential interaction of VIMP with an ERAD complex containing HERP and HRD1. Although VIMP coprecipitated with overexpressed ZZ-tagged HERP, this interaction was not observed using antibodies specific for HERP and HRD1 to precipitate the complex. Consistent with these results the interaction of VIMP with a viral E3 ubiquitin ligase, mK3, could hardly be detected, although mK3 clearly associates with Derlin-1 and p97 to mediate the degradation of MHC class I molecules (Wang et al., 2006). Thus, it is feasible that VIMP forms another complex with Derlin-1 and p97 in which HRD1 and HERP are not present. VIMP is also upregulated upon ER stress (Gao et al., 2004) and associates with p97 and Derlin-1 (Ye et al., 2004), which argues in favour of a role for VIMP in ERAD. However, its function can no longer be restricted to linking p97 to the ER membrane, as suggested by Ye and coworkers (Ye et al., 2004). Interestingly, VIMP was identified as a novel selenoprotein and therefore named SelS (Gao et al., 2004). Other selenoproteins, e.g. gluthathione peroxidases or thioredoxin reductases, regulate the cellular redox balance and therefore have antioxidant properties. As VIMP was also shown to protect cells from oxidative stress (Gao et al., 2004), it might have a similar role. It might participate in the unfolding of ERAD substrates at the cytoplasmic side of the ER membrane by breaking the disulfide bonds of ERAD substrates. This indicates that VIMP functions in a late step within ERAD downstream of the action of HERP and HRD1.

4.3.2 p97 is recruited to the ER membrane via multiple interactions

It was reported that the integral membrane protein VIMP mediates the association of p97 to the ER membrane (Ye et al., 2004). However, this study has revealed two further integral membrane proteins, which interact with p97 and therefore recruit p97 to the ER membrane: HRD1 as well as Derlin-1 were both shown to directly interact with p97. Additionally, in yeast the ER resident protein Ubx2p was demonstrated to bind the p97 homologue Cdc48p (Neuber et al., 2005; Schuberth and Buchberger, 2005). These data suggest that p97 is tethered to the ER membrane via multiple interactions. All these interactions contribute to the close association of this soluble ERAD component with the ER membrane. Ubx2p was also shown to mediate the recruitment of Cdc48p to certain ERAD components such as the yeast ERAD E3 ligases Hrd1p and Doa10p, the Derlin-1 orthologue Der1p and to ERAD substrates (Neuber et al., 2005; Schuberth and Buchberger, 2005). This demonstrates that the close cooperation of the ubiquitylation machinery and the retrotranslocation factors appears to be conserved from yeast to man. A mammalian homologue of the yeast Ubx2p exists and future experiments will certainly demonstrate its involvement in ERAD.

The p97 hexamer appears to have a central position within this complex. It associates with a number of integral membrane proteins, but also with cytosolic proteins that have a role in ERAD. In association with the cofactors UFD1 and NPL4 the p97 hexamer was considered to be a key component for retrotranslocation of substrate proteins (Bays et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). The cofactors, UFD1 and NPL4, contain polyubiquitin binding sites (Ye et al., 2003), which together with those on UBX2 and p97 might ensure the directionality of substrate transport. ERAD components capable of binding polyubiquitin reside at the membrane near the site of ubiquitylation and presumably prevent the backsliding of ubiquitylated ERAD substrates into the ER lumen. Interestingly, UFD1 cannot be coprecipitated with HRD1 (M. Seeger, personal communication), indicating that the p97-cofactors UFD1 and NPL4 bind the complex after HRD1 and HERP have already dissociated from the complex.

Apart from the p97-UFD1-NPL4 complex the proteasome itself was also proposed to participate in retrotranslocation of ER proteins. In fact, the model substrate pro-α-factor in yeast can be extracted from the ER membrane *in vitro* in an ATP-dependent manner using purified 26S proteasomes, or even 19S cap particles alone (Lee et al., 2004). However, since pro-α-factor degradation is independent of polyubiquitylation, its degradation differs mechanistically from that of other ERAD substrates. In addition, in my experiments the proteasome was not

coprecipitated with any of the ERAD components tested. Thus, it appears not to be associated with the ERAD complex containing p97, Derlin-1, HRD1 and HERP.

4.3.3 The channel question

It was initially proposed that retrotranslocation of ERAD substrates occurs through the Sec61 channel, which also mediates the import of nascent proteins into the ER. In cells which express the HCMV protein US2, partially dislocated MHC class I molecules were precipitated with a subunit of the Sec61 channel (Wiertz et al., 1996). Similarly, the ERAD target ApoB100 was crosslinked with the Sec61 channel (Pariyarath et al., 2001). Experiments in yeast have also linked Sec61 to protein export from the ER (Pilon et al., 1997; Plemper et al., 1997; Plemper et al., 1998). Accordingly, the Sec61 channel is upregulated by the UPR (Travers et al., 2000). Recent data also demonstrate an interaction of the 26S proteasome with Sec61. In this study the proteasome was shown to compete with the ribosome for Sec61 binding (Kalies et al., 2005). Since there is evidence that the proteasome itself can also perform retrotranslocation of ERAD substrates (Lee et al., 2004), there might indeed be a direct route from the import channel Sec61 to the proteasome.

In contrast to these findings several reports argue against an involvement of the Sec61 complex in ERAD. This import channel was, for instance, not coprecipitated with Protein A-tagged Ubx2p in yeast, although Cdc48p, Doa10p and Hrd1p clearly interacted with Ubx2p (Neuber et al., 2005). Furthermore, several lines of evidence support the idea that Derlin-1 and its paralogues form a retrotranslocation pore. Derlin-1 has two human paralogues designated as Derlin-2 and Derlin-3, and all of them have been described in detail (Lilley and Ploegh, 2005; Oda et al., 2006; Ye et al., 2005). They were all shown to be upregulated by the UPR and to associate with p97. Additionally, Derlin-2 and Derlin-3 were found to associate with EDEM, an ER-resident lectin responsible for substrate recognition (Oda et al., 2006). This indicates that Derlin-2 and -3 appear to associate with ERAD components on both sides of the ER membrane. Derlin-2 was also reported to bind Derlin-1 (Lilley and Ploegh, 2005), whereas Derlin-1 was shown to form homooligomers (Ye et al., 2005). The oligomeric nature of the Derlin proteins and the capacity of Derlin-1 to comigrate with early retrotranslocation intermediates in sucrose gradients (Ye et al., 2005) suggests that the Derlin proteins might be part of the retrotranslocation pore. However, because the ubiquitin ligases HRD1 and gp78 are also multispanning membrane proteins, it is possible that they contribute to the formation of a channel. In support of this hypothesis a direct interaction of HRD1 with Derlin-1 was observed in the study presented here.

Experiments in yeast demonstrated that certain membrane proteins do not require Der1p, the yeast homologue of Derlin-1, for retrotranslocation (Knop et al., 1996; Vashist and Ng, 2004). These substrates might use another retrotranslocation channel, possibly the Sec61 channel, or are extracted from the ER membrane without a channel. Thus, within ERAD different substrate-specific pathways might exist to guarantee the efficient elimination of ER proteins by the proteasome. Newly synthesised ER proteins, which are still attached to the Sec61 channel, might use this import channel for retrotranslocation and the proteasome for both extraction and degradation. Other ERAD substrates, however, might rely on an ERAD complex containing HERP, HRD1, Derlin-1, p97 and other proteins.

4.3.4 Shuttle proteins for the proteasome within ERAD

Since many UDPs have been shown to interact with the proteasome before, it was tempting to speculate that the UDP HERP also interacts with the proteasome, thereby recruiting the degradation machinery to the ER membrane, the site of ERAD. However, my results clarified that HERP does not share the proteasome binding properties of other UDPs. Additionally, the proteasome was not coprecipitated with any of the ERAD components tested in this study.

Recently, the yeast E4 enzyme Ufd2p was shown to target the UBL/UBA proteins Rad23p and Dsk2p (Kim et al., 2004). Since Ufd2p was also known to associate with Cdc48p (p97) (Koegl et al., 1999), this indicated a route from Cdc48p via Ufd2p and the UBL/UBA proteins to the proteasome. In fact, a pathway was described in which oligoubiquitylated substrates are first collected by the Cdc48p complex with its cofactors Ufd1p and Npl4p, subsequently polyubiquitylated by the E4 enzyme Ufd2p and finally bound by Rad23p/Dsk2p for proteasomal targeting (Richly et al., 2005). More evidence for a function of UBL/UBA proteins in ERAD came from genetic experiments in yeast showing that Rad23p and Dsk2p are essential for the degradation of proteins derived from the ER (Medicherla et al., 2004). Thus, the UBL/UBA proteins Dsk2p and Rad23p are not only required for recruiting cytosolic ubiquitin conjugates to the proteasome. They also shuttle ER-derived substrates.

The human counterparts of these proteins, UFD2 and HHR23B, also interact with each other (data not shown), indicating that the general mechanism of the recruitment of ERAD substrates to the proteasome is conserved between species. However, this study demonstrated that HHR23B is not associated with p97 or other ERAD components upstream of the proteasome. Thus, HHR23B might bind UFD2 after the E4 enzyme has dissociated from p97.

Alternatively, the murine deglycosylating enzyme PNGase also connects p97 and HR23B and there is evidence that UFD2 and PNGase define two distinct HR23B dependent pathways (Biswas et al., 2004; Katiyar et al., 2004; Kim et al., 2006; Li et al., 2005). Thus, HR23B uses different cofactors to escort substrate proteins to the proteasome for degradation.

4.3.5 HERP and HRD1: a functional unit?

Certain UDPs were shown to interact with E3 ligases or even display E3 ligase activity themselves. Examples are KPC2, ElonginB, BAG1 and parkin. This study revealed another interaction of that kind: The UDP HERP was shown to directly interact with the E3 ligase HRD1. Additionally, both proteins strikingly comigrated in glycerol gradients. This suggested that HERP and HRD1 closely cooperate in the disposal of unfolded proteins within ERAD. When HRD1 was overexpressed in HeLa cells, HERP was stabilised, as demonstrated with a cycloheximide chase experiment. This stabilisation was independent of the HRD1 RING domain, since overexpression of a RING mutant version of HRD1 also resulted in HERP stabilisation.

Apart from this observation, increased amounts of Derlin-1 and p97 seemed to migrate in fractions of higher density in the gradients with overexpressed wildtype and mutant HRD1 compared to mock-transfected cells. This indicated that overexpression of the E3 ligase results in the formation of more ERAD complexes leading to larger amounts of p97 and Derlin-1 in the fractions of higher density. This finding might also explain the higher stability of HERP when HRD1 is overexpressed. HERP stability is dependent on HRD1 association possibly because both proteins are only functional when they are associated with each other. When larger amounts of HRD1 are available HERP levels also have to be increased. Normally, synthesis of both proteins is upregulated by the same pathway so that equal proportions of both proteins are guaranteed. The overexpression of HRD1 leads to an imbalance of the two proteins which is compensated by the stabilisation of HERP.

These data establish that HERP and HRD1 indeed cooperate in the elimination of misfolded proteins from the ER. The precise molecular function of HERP could not be solved here and further studies are needed to elucidate whether HERP is required for ubiquitylation or for retrotranslocation of ERAD substrates. However, its interaction with the DUB USP7 points out a role in regulating the balance between ubiquitylation and deubiquitylation, as will now be discussed in further detail.

4.3.6 The interaction of HERP and USP7 suggests a role for HERP in ERAD

In the study presented here two proteins, the E3 ligase HRD1 and the deubiquitylating enzyme USP7, were identified as direct interaction partners of the UDP HERP. The HERP interaction with HRD1 was mapped to a region downstream of the HERP UBL domain, while HERP was shown to interact with USP7 in a UBL domain dependent manner. Since different domains are required for these interactions, it is feasible that HERP can form a ternary complex with HRD1 and USP7. These findings pose new questions: Why is HERP associated with potentially antagonistic functions of ubiquitylation and deubiquitylation? Its interaction with USP7 indicates that deubiquitylation is also an important aspect within ERAD. However, what is deubiquitylation needed for in the course of ERAD?

Apart from the activation of ubiquitin precursors, DUBs generally have two major functions in the cell: One function is to maintain the cellular pool of free ubiquitin by cleaving polyubiquitin chains from substrates, which have been targeted to the proteasome and committed to degradation. This function is carried out by ubiquitin COOH-terminal hydrolases (UCHs), which are small proteins, usually less than 30 kDa, some of which are associated with the 26S proteasome. This guarantees that substrate proteins are degraded by the proteasome while ubiquitin is recycled in a new ubiquitylation reaction. Another function of DUBs is to reverse ubiquitylation of specific substrates, hence, reverse their fate. This function is generally performed by a second class of DUBs, the ubiquitin-specific proteases (USPs), one of which is the DUB USP7. In fact, a role for USP7 in deubiquitylation and thereby stabilisation of p53 has been described (Li et al., 2002). Is it possible that USP7 reverses the fate of ERAD substrates by their deubiquitylation? A recent report by Rumpf and Jentsch argues in favour of this concept. They demonstrated that the deubiquitylating enzyme Otu1p inhibits the Ufd2p-Rad23p-mediated degradation of the NF-κB-related transcription factor, the first identified natural Cdc48p substrate, namely Spt23p (Rumpf and Jentsch, 2006). Thus, ubiquitin ligase activity can indeed be counteracted by the action of a deubquitylating enzyme.

A similar function could also be envisaged for USP7. However, the findings observed in this study argue against such a role. If USP7 would reverse the fate of ERAD substrates, then the siRNA-mediated downregulation of HERP would result in an accelerated CD3-delta degradation. However, the opposite was the case. Thus, USP7 might alternatively be required for the deubiquitylation of ERAD substrates once they are committed to proteasomal degradation, thereby contributing to the pool of free ubiquitin (Fig. 32A).

Interestingly, USP7 has also been implicated in protecting a viral E3 ligase called ICP0 from autocatalysed ubiquitylation and degradation. USP7 was shown to interact with ICP0 and, although this did not affect the activity of ICP0 *in vitro*, their interaction greatly enhanced the stability of ICP0 *in vivo*. ICP0-mediated autoubiquitylation was reversed by the activity of USP7, which specifically removed polyubiquitin chains from the E3 ligase (Canning et al., 2004). It is feasible that USP7 has a related role in the course of ERAD, too. Additionally, since HERP was shown to interact with various E3 ligases (data not shown), HERP might protect these E3 ligases from autocatalysed ubiquitylation and degradation by recruiting USP7 to E3 ligases. With regard to the ERAD complex, it is likely that HRD1 and other ERAD ubiquitin ligases ubiquitylate other ERAD components and not just themselves. Hence, by recruiting USP7 to the complex HERP might protect various ERAD components from ubiquitylation by E3 ligases and thereby degradation (Fig. 32B). Confusingly, a HERP version which lacked the UBL domain was shown to be more stable compared to full length HERP, although the UBL domain was shown not to contain the lysine residue important for ubiquitylation (Sai et al., 2003). These findings argue against this hypothesis. However, since these findings could not be verified in this study (data not shown), its remains to be seen, whether the UBL domain of HERP has an impact on HERP stability.

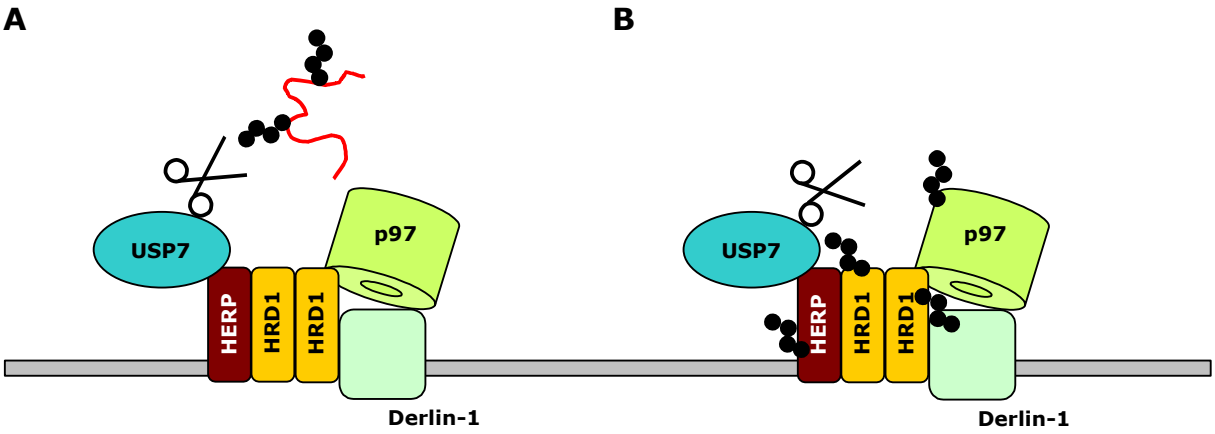

Fig. 32. Two alternative models for potential roles of HERP and USP7 in ERAD. (A) HERP recruits USP7 to the ERAD complex, which deubiquitylates ERAD substrates prior to their degradation by the 26S proteasome. **(B)** Since E3 ligases tend to ubiquitylate themselves and other proteins in proximity, USP7 might be required to rescue these proteins from proteasomal degradation by reversing ubiquitylation.

To eluciate the role of USP7 within ERAD, an siRNA-mediated downregulation of USP7 as well as a dominant negative version of USP7 containing solely the N-terminus of USP7 and not the UCH domain were established. Using these tools the influence of USP7 on the degradation of several ERAD components and the ERAD substrate CD3-delta can be monitored. This experiment will sure reveal the exact role of USP7 within ERAD.

4.3.7 A model for the ERAD pathway

In summary, the observations presented here in combination with data from other groups allow to propose a model for the ERAD pathway. In an initial step Derlin-1 and its paralogues associate with ERAD substrates and prepare them for export out of the ER (Fig. 33). This results in the recruitment of E3 ligases and p97, which both perform direct interactions with Derlin proteins.

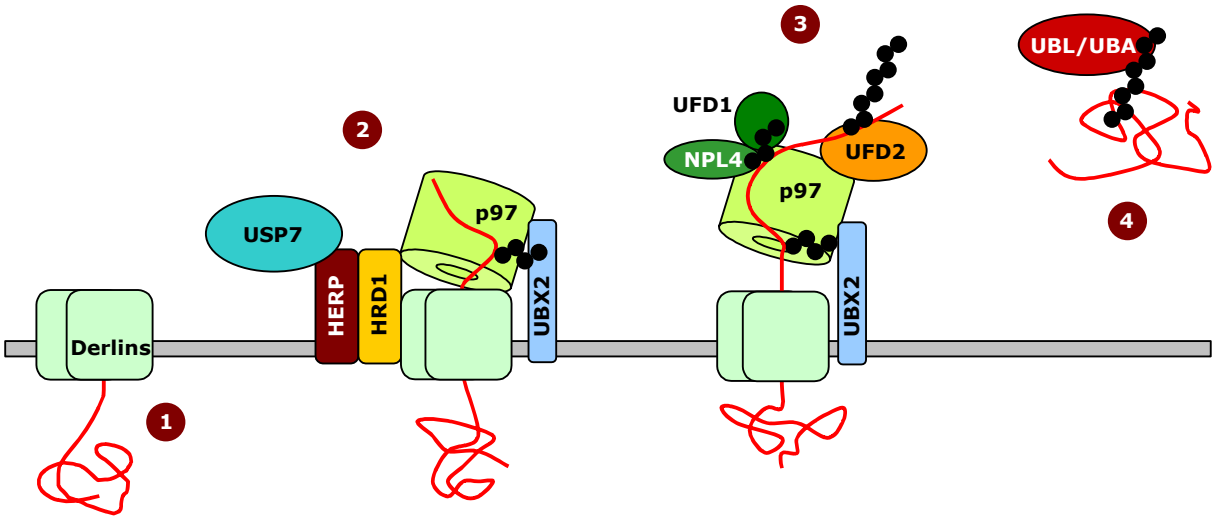

Figure 33. Model for the assembly of the ERAD complex. (1) Derlin-1, -2 or -3 might first associate with ERAD substrates and prepare substrates for export out of the ER. **(2)** Retrotranslocation of substrates also requires the association of E3 ligases such as HRD1 and/or gp78. These E3 ligases might contribute to the formation of the channel and conduct ubiquitylation of substrate proteins at the cytoplasmic side of the ER membrane. HERP recruits USP7 to the complex, which rescues other ERAD components from ubiquitylation by E3 ligases. **(3)** After dissociation of HERP and HRD1 the two cofactors NPL4 and UFD1 bind to p97 to facilitate retrotranslocation of ERAD substrates. Ubiquitin-binding sites on p97, UFD1, NPL4 and UBX2 prevent the backsliding of substrate proteins into the ER. VIMP and PNGase perform unfolding and deglycosylation of ER proteins (not shown in the model) whereas UFD2 permits polyubiquitylation of oligoubiquitylated substrates. Retrotranslocation is completed by p97-Ufd1-Npl4 and **(4)** released ERAD substrates are conveyed to the proteasome by UBL/UBA proteins such as HHR23B.

E3 ligases such as HRD1 and gp78 contribute to the formation of the retrotranslocation channel and thus mediate both retrotranslocation as well as ubiquitylation of ERAD substrates. The ATPase p97 is already associated with the early ERAD complex to facilitate substrate export. Substrates are retrotranslocated and ubiquitylated once they reach the cytosolic side of the ER membrane. HERP is always associated with the E3 ligases and recruits USP7 to rescue E3 ligases from autoubiquitylation and ERAD components from ubiquitylation by E3 ligases. UBX2 might already be associated with the complex. However, other ubiquitin-binding factors like UFD1 and NPL4 bind later, after E3 ligases have disassembled from the complex. These polyubiquitin-binding factors might prevent the backsliding of substrate proteins into the ER. It is likely, that after ubiquitylation is accomplished the p97-UFD1-NPL4 complex dissociates from the ER membrane to release ubiquitylated substrates into the cytosol. VIMP and PNGase might be involved in breaking disulfide bonds and deglycosylation, respectively, while UFD2 performs polyubiquitylation of oligoubiquitylated substrates. Concomitantly, these substrates are recognised by UBL/UBA proteins such as HHR23B or Ubiquilin-1, which recruit the substrates to the 26S proteasome for degradation. The model presented here is most likely not complete and further yet unidentified proteins might also be involved in the elimination of misfolded proteins from the ER. Hence, future experiments will certainly reveal novel components implicated in this finely tuned process of ER-associated protein degradation.

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ABBREVIATIONS

amp	ampicillin
BAG	BCL2-associated athanogene
BSA	bovine serum albumin
CFTR	cystic fibrosis transmembrane conductance regulator
DBC	DeoxyBigChap
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
e. g.	*exempli gratia* = for example
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERSE	ER stress response element
FCS	fetal calf serum
GST	Glutathione S-transferase
g	acceleration of gravity
fwd	forward
HECT	homologous to the E6AP carboxyl terminus
HEPES	1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt
HERP	homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain protein
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
Ig	immunoglobulin
IPTG	isopropyl-beta-D-thiogalactopyranoside
HRD	HMG-CoA reductase degradation
IP	immunoprecipitation
kan	kanamycin
kb	kilobases
kDa	kilodaltons
LB	Luria Bertani
M	mol/L
MATH	meprin and TRAF homology
MHC	major histocompatibility complex
MUBL	membrane resident UBL domain containing protein
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rev	reverse
RING	really interesting new gene
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S	Svedberg, sedimentation coefficient
SCF	Skp1-Cullin-F-box
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TCA	trichloroacetic acid
siRNA	small interfering RNA
TEMED	tetramethyl ethylene diamine
Tris	Tris (hydrodxymethyl) aminomethane
TEV	Tobacco Etch Virus
UCH	ubiquitin COOH-terminal hydrolase
U	unit
UBL	ubiquitin-like
UDP	ubiquitin domain protein
UFD	ubiquitin fusion degradation
USP	ubiquitin specific protease
VIMP	VCP-interacting membrane protein

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- **Andrea Schulze**, Rasmus Hartmann-Petersen und Michael Seeger. Ubiquitin Domain Proteins - Functional Variations of a Common Structure. In *The Ubiquitin Proteasome System in the Central Nervous System - from Physiology to Pathology*. NovaScience Publisher. Editoren: Mario Di Napoli und Cezary Wojcik. Zur Publikation angenommen.

- **Andrea Schulze**, Sybille Standera, Elke Bürger, Marjolein Kikkert, Sjaak van Voorden, Immanuel Wiertz, Peter-Michael Kloetzel und Michael Seeger (2005). The Ubiquitin Domain Protein HERP Forms a Complex with Components of the Endoplasmic Reticulum Associated Degradation Pathway. *Journal of Molecular Biology* 354, 1021-1027.

- Vasily Rybakin, Maria Stumpf, **Andrea Schulze**, Irina V. Majoul, Angelika A. Noegel und Andreas Hasse (2004). Coronin 7, the Mammalian POD-1 Homologue, Localizes to the Golgi Apparatus. *FEBS Letters* 573, 161-167.

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Posterbeiträge

- **Andrea Schulze**, Sybille Standera, Elke Bürger, Peter-Michael Kloetzel und Michael Seeger. Structural and Functional Interplay of the Two ERAD Components HERP and HRD1. *Cold Spring Harbor Meeting: The ubiquitin family, Cold Spring Harbor, New York,* 27.04.-01.05.2005.

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DANKSAGUNG

Besonderer Dank gilt Herrn Prof. Dr. Peter-Michael Kloetzel, der es mir ermöglichte, in seiner Arbeitsgruppe unter hervorragenden Bedingungen zu arbeiten und zu diskutieren.

Ganz besonders bedanke ich mich auch bei Herrn Dr. Michael Seeger für die interessante Themenstellung, die Betreuung der Arbeit und die zahlreichen Tipps und Anregungen, sowie seine ständige Diskussionsbereitschaft. Seine wissenschaftliche Unterstützung war ein entscheidender Beitrag zu dieser Arbeit.

Herrn Prof. Dr. Wolfgang Dubiel und Herrn Prof. Rasmus Hartmann-Petersen, PhD gilt mein Dank für die Begutachtung der Arbeit. Außerdem möchte ich mich bei den Kooperationspartnern Dr. Marjolein Kikkert (Leiden University Medical Center), Dr. Madleon Maurice (University Medical Center Utrecht), Dr. Yihong Ye und Prof. Dr. Tom Rapoport (beide Harvard Medical School, Boston) für die wertvollen Diskussionen und die Bereitstellung verschiedener Materialien bedanken.

Bei Sybille Standera und Elke Bürger bedanke ich mich für die hervorragende technische Unterstützung und für die vielen Tipps und Tricks hinsichtlich der Laborpraxis. Auch Annett Helfrich gilt mein Dank für die gute Zusammenarbeit. Ein herzliches Dankeschön geht an Ulf Gurok, Melanie Dyllick-Brenzinger, Gesa Hoffmann, Britta Strehl und Melanie Kny für das Korrekturlesen der Arbeit.

An dieser Stelle sei auch allen Mitarbeitern und Mitarbeiterinnen der Arbeitsgruppe Kloetzel für Ihre Hilfsbereitschaft und für das unkomplizierte und gute Arbeitsklima gedankt. Bei der gesamten Besatzung des Doktorandenraums bedanke ich mich für die vielen Aufmunterungen und Diskussionen. Besonders möchte ich dabei Britta Strehl, Gesa Hoffmann, Melanie Rieger und Benjamin Fricke erwähnen.

Nicht zuletzt bin ich sehr dankbar für die Gewährung meines Promotionsstipendiums durch die Charité.

ERKLÄRUNG

Hiermit versichere ich, die Dissertation selbständig und ohne unerlaubte Hilfe angefertigt zu haben.

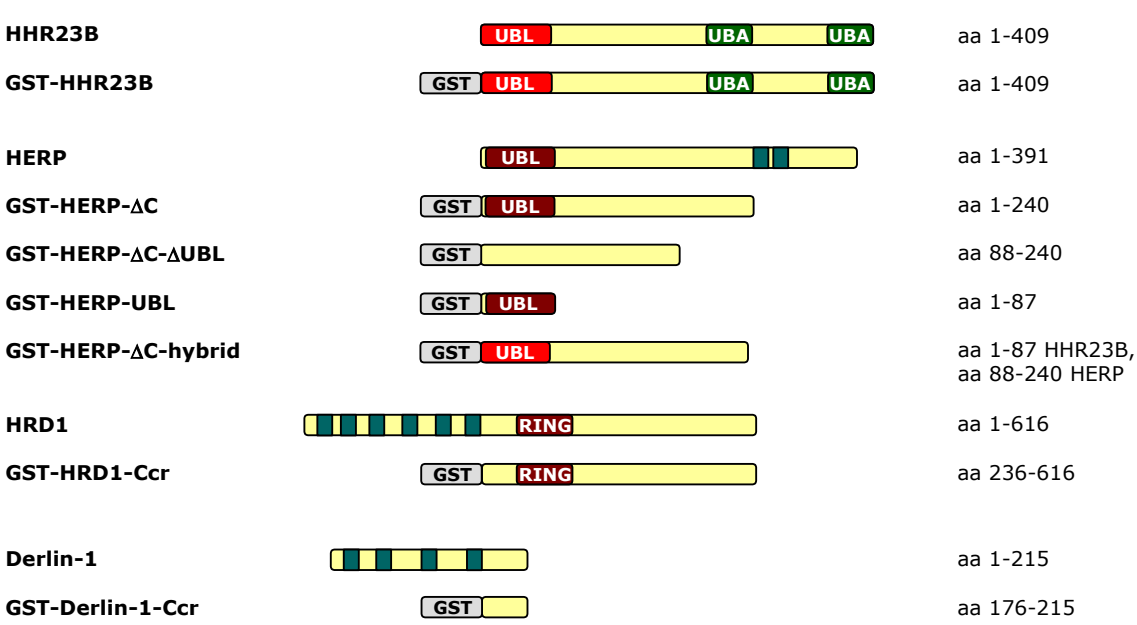
Berlin, April 2006

Andrea Schulze

APPENDIX

GST- and ZZ-fusion proteins used in this study:

GST-fusions:



ZZ-fusions: